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A FACTOR FROM NORMAL TISSUES INHIBITING TUMOR GROWTH*

BY JAMES B. MURPHY, M.D., AND ERNEST STURM

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, June 21, 1934)

A factor has been demonstrated in certain tumors of the fowl which has a definite inhibiting or neutralizing action on the transmitting agents of these tumors (1). It has proved to be non-specific in its effect, as shown by the fact that it will slow down or actually prevent the growth of a mouse sarcoma (2). It has been suggested that this factor may be related to the control or balancing mechanism of normal cells. The present paper is a report of an investigation designed to test the possible presence of a growth-inhibiting factor in active normal tissues (3).

It might be expected that tissues with the greatest growth energy would require a greater concentration of the hypothetical balancing factor to insure controlled and regulated growth. Therefore, the embryonic tissues have been utilized as the main source of material investigated.

Material and Method

Several strains of standard transplantable mouse tumors have served as the medium on which the growth-inhibiting factors were tested, the principal ones being the Bashford Adenocarcinoma No. 63, Carcinoma 48, and Sarcoma 180.

Following the method which had given the best yield of the inhibiting substance from the fowl tumors, desiccates of the tissue were employed. The tissues or organs to be studied were removed, minced finely, spread in a thin layer, and placed immediately in a vacuum jar over sulfuric acid. After being evacuated down to about 1 mm. mercury the container was placed in a freezing box and allowed to remain until desiccation was complete. The dry flakes were ground to a fine powder and stored in sealed tubes at ice box temperature until used. The test solutions were prepared by thoroughly extracting 0.1 gm. of the desiccate with 2 cc. of water, by pumping back and forth in a syringe through a coarse needle.

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* This investigation was carried out under the Rutherford Donation.

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The larger particles were centrifuged out and generally the supernatant fluid was heated at 48°C. for 30 minutes. The Bashford tumor was cut into generous sized grafts and these were nicked in several places to give a greater area of exposed surface. Half of these were placed in the test solution and half in normal salt solution. The time of contact was only that required for loading the grafts into trocars. A certain amount of the extract was carried along with the inoculated material. With No. 48 and the sarcoma, cell suspensions were generally used for inoculation. These were prepared by pressing the tumor through a fine wire mesh, and half of the *Brei* was suspended in the tissue extract and the other half in normal salt solution in a ratio of 1:1 between cells and suspending fluid. From 0.05 to 0.1 cc. of these suspensions were injected. In order to secure a more complete check each animal inoculated with the tumor and tissue extract in one groin received the control inoculation of the tumor in salt solution in the other groin.[1] Besides these controls additional animals were frequently inoculated in both groins with the same tumor material in salt solution.

TABLE I

Effect of Extracts of Mouse Placenta and Embryo Skin on Mouse Tumor Bashford 63

Material inoculated with Bashford tumor graft	No. of experiments	No. of mice inoculated	No. of tumors	Average size of tumors cm.	No. negative	Per cent negative
Extract of dry mouse placenta	19	225	91	1.02 x 0.82	134	59.6
Salt solution (grafts inoculated in same animals as above)		211	163	1.43 x 1.10	48	22.7
Salt solution (grafts inoculated in other animals)		110	109	1.73 x 1.36	18	16.4
Extract of dry embryo skin	10	144	64	1.09 x 0.97	80	55.5
Salt solution (grafts inoculated in same animals as above)		84	66	1.38 x 1.08	18	21.4
Salt solution (grafts inoculated in other animals)		90	69	1.66 x 1.36	25	27.7
Extract of fresh placenta or embryo skin	8	79	56		23	29.1
Salt solution		70	40		30	40.2

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[1] It was perhaps not sufficiently emphasized in our earlier papers on the inhibitors in the fowl tumors that the test and the control materials were both inoculated in each fowl, using the intradermal site where accurate measurements may be obtained. By having each test controlled in the same animal the natural variation in degree of susceptibility is eliminated in judging the results.

The Effect of Homologous Tissue Extracts on the Growth of Transplantable Tumors

The first investigation was carried out with various homologous tissues, but only two strains of tumors were used as indicators.

Experiment.—Fresh tissues were utilized for the earlier experiments and they included fresh mouse placenta, embryo skin, and skinless embryos, with the extracts both heated and unheated. These extracts tested by 222 inoculations of the Bashford 63 failed to show any detectable effect on either the number of takes or the growth rates of the resulting tumors. Likewise, extracts of desiccated skinless embryos and mouse blood, tested by 102 inoculations of the same tumor, showed no influence on the resulting tumors as compared with the controls.

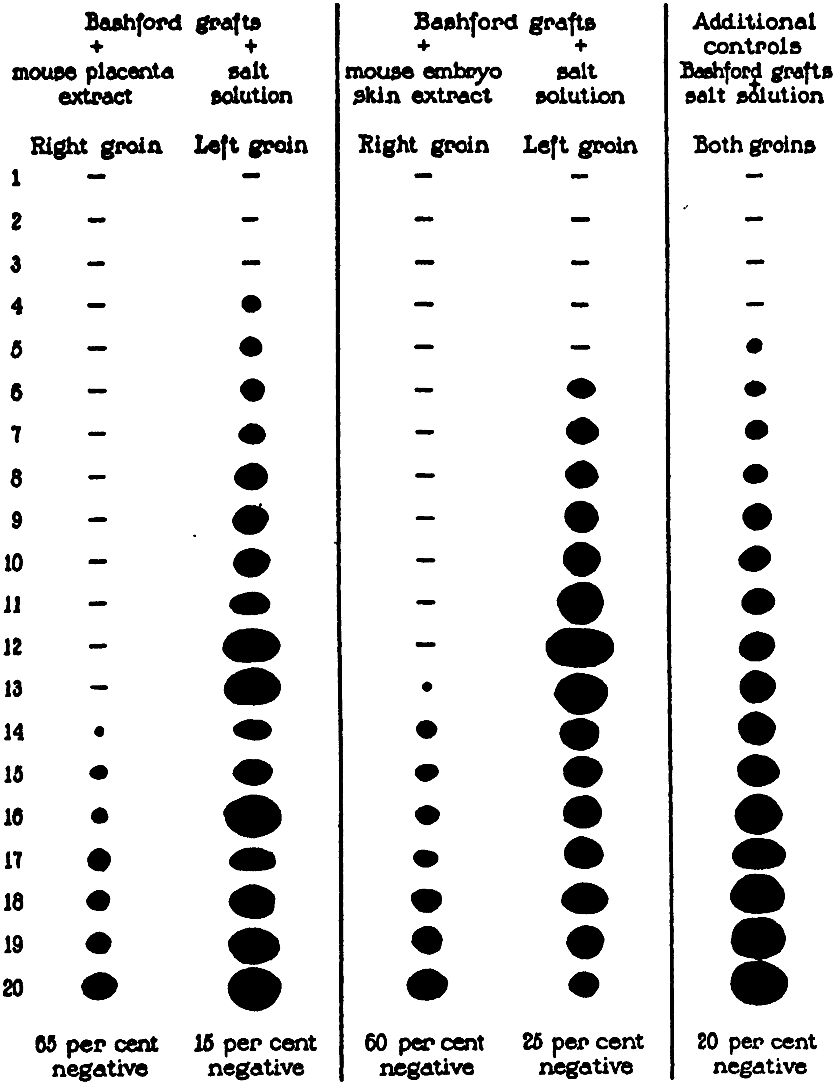
The major experiments were directed towards testing extracts of desiccated mouse placenta and embryo skin, as preliminary tests had indicated an effect on tumors by extracts of these two tissues.

The methods used were those described above. The length of contact between the tumor graft and the solutions was at the maximum 20 minutes. As the heating of the extract at 48°C. for 30 minutes had no material effect on the result all of the experimental results are grouped together. In the majority of instances the control material of tumor immersed in salt solution for a corresponding time was inoculated into the same animal, as well as in other animals. The results, including the average size of the tumor, are given in Table I.

The outcome of a typical experiment is shown in Text-fig. 1.

A similar test was carried out on the effect of the embryo skin and placenta extracts on Mouse Sarcoma 180. The results of these are given in Table II.

The extracts of desiccated mouse placenta and mouse embryo skin give evidence of inhibiting the growth of the Bashford Mouse Tumor 63. The test involved 369 inoculations controlled by 495 inoculations of the same tumor and the effect is shown not only by the material reduction in the number of takes of the treated grafts but by the slow rate of growth of such tumors as were not completely suppressed. In fact there were very few instances in which the tumor arising from the treated graft was not definitely smaller than that from the control graft inoculated into the same animal. There was an absolute failure of extracts prepared from fresh placenta or embryo skin to produce any effect on the growth of this tumor. The Mouse Sarcoma 180 which was definitely retarded by the inhibitor from chicken tumors (2) was unaffected by extracts either from fresh or desiccated mouse tissues.



Text-Fig. 1. Effect of homologous tissue extracts on mouse tumor.

Attempts to Induce General Resistance

While there is nothing in the above described experiments to suggest that the results are in any way connected with the well known induced resistance to transplantable tumors, it seemed advisable to test this point. The method by which this resistant state brought about is

TABLE II

Effect of Homologous Tissue Extracts on Mouse Sarcoma 180

Material inoculated with Mouse Tumor 180	No. of experiments	No. of mice inoculated	No. of tumors	Average size of tumors	No. negative	Per cent negative
				cm.		
Extract of dry mouse placenta	10	86	85	2.05 x 1.75	1	1.2
Salt solution		69	69	2.20 x 1.90	0	0.0
Extract of dry embryo skin	5	49	49	1.70 x 1.30	0	0.0
Salt solution		50	50	1.70 x 1.20	0	0.0
Extract of fresh placenta	2	12	12	1.80 x 1.55	0	0.0
Salt solution		12	12	2.10 x 1.70	0	0.0
Extract of dry skinless embryo	7	59	59	2.35 x 1.85	0	0.0
Salt solution		47	47	2.55 x 2.15	1	2.0
Extract of fresh skinless embryo	2	12	12	2.20 x 1.00	0	0.0
Salt solution		12	12	2.10 x 1.70	1	0.0

TABLE III

Effect of Previous Injection of Tissue Extracts on the Growth of Tumors

	No. of experiments	No. of mice inoculated	No. negative	Per cent negative
0.2 cc. placenta or embryo skin extract injected 7 days prior to inoculation of Bashford tumor	4	44	14	31.8
Bashford tumor alone (control)		34	8	23.5
0.2 cc. placenta of embryo skin extract injected 7 days prior to inoculation of Sarcoma 180	2	12	1	8.3
Sarcoma 180 alone (control)		18	0	0.0

the injection of homologous living normal cells into the animal 7 to 10 days prior to the inoculation of the tumor.

Experiment.—Based on four experiments, 44 mice were given subcutaneously 0.2 cc. of either an extract of dry placenta or dry embryo skin, and 7 days later these mice were inoculated with grafts of the Bashford tumor. At the same time 34 controls were inoculated with the same tumor. A similar experiment was carried out on mice subsequently inoculated with Sarcoma 180. The results of these tests are given in Table III.

There is no evidence from these experiments that extracts of desiccated placenta or embryo skin induce a subsequent general resistance to inoculated tumor.

TABLE IV

Effect of Heterologous Tissue Extracts on the Bashford Tumor

Material inoculated with Bashford 63	No. of experiments	No. of mice inoculated	No. of tumors	Average size of tumors	No. negative	Per cent negative
				cm.		
Extract of desiccated rabbit placenta	6	68	23	1.52 x 1.30	45	66.2
Salt solution		68	47	1.97 x 1.40	21	30.9
Extract of desiccated rat placenta	5	59	17	1.10 x 0.90	42	71.1
Salt solution		59	43	1.60 x 1.20	16	27.1
Extract of desiccated rat embryo skin	4	40	18	0.90 x 0.70	22	55.0
Salt solution		40	26	1.40 x 1.00	12	30.0
Extract of fresh rabbit placenta	2	17	14	1.32 x 0.95	3	17.6
Salt solution		17	13	1.30 x 1.05	4	23.5

Inhibiting Action of Heterologous Tissue Extracts

As noted above, the inhibitors from chicken sarcomas are not species limited in their action, as shown by their effect on mouse sarcoma. In the next group of experiments we tested heterologous tissue extracts on transplantable mouse tumors.

Experiment.—In a series of 293 inoculations, controlled by 283 inoculations, the following heterologous tissue extracts showed no very definite effect on either the Bashford Tumor 63 or Carcinoma 48; desiccated and fresh calf thymus, desiccated kidney and spleen of the rat and rabbit, early and term human placenta, and early cow, rabbit, and hog placenta.

The main experiments were tests of rat and rabbit placenta and rat embryo skin. The methods employed were those described above. The materials were collected during the later stages of pregnancy. The data from tests on Bashford tumor grafts are given in Table IV, and for cell suspensions of Tumor 48 in Table V. The results of a typical experiment are shown in Text-fig. 2.

The inhibiting action of rat placenta and embryo skin and of rabbit placenta is quite definite. As in the case of the homologous tissues the action is manifest not only by the complete suppression of growth in a high percentage, but where the suppression was not complete the growth rate of the resulting tumors was almost invariably distinctly slower than that shown by the control grafts in the same animals. These points are well shown by Text-fig. 2. The failure of the extracts of fresh tissue are just as evident with heterologous as with homologous tissues. There was no definite effect from extracts of desiccated early cow, hog, or human placentas or from human placentas at term.

TABLE V

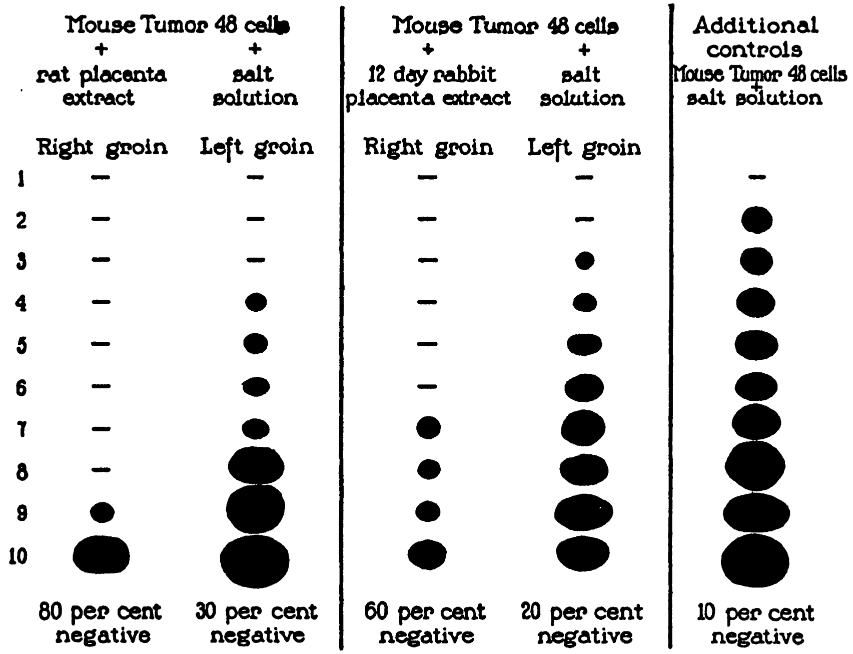
Effect of Heterologous Tissue Extracts on Mouse Carcinoma 48

Material inoculated with cell suspension of Mouse Tumor 180	No. of experiments	No. of mice inoculated	No. of tumors	Average size of tumors cm.	No. negative	Per cent negative
Extract of desiccated rabbit placenta	28	275	114	1.19 x 0.92	161	58.5
Salt solution		275	220	1.58 x 1.14	55	20.0
Extract of desiccated rat placenta	2	20	7	1.40 x 1.00	13	65.0
Salt solution		20	14	1.50 x 1.10	6	30.0

The Effect of Age of Placenta on Inhibiting Action

The absence of any very definite inhibiting action of the extracts of early hog, cow, and human placentas and of human placentas at term opened the question as to whether this was due to the gap between the species or to the age of the placentas. We had some preliminary evidence that early stages of rabbit placenta were not active, while the later stages were active. On the basis of this evidence of a possible bearing of the age of the placenta on its inhibiting action, a systematic investigation of the point was taken up.

Experiments.—Rabbit placentas were collected at the 12th, 15th, 19th, 22nd, 26th, and 28th day of pregnancy (30 day term) and prepared in the way described above. The extracts of the desiccates were tested on Mouse Tumor 48 in the usual way, with control inoculations of the tumor suspension in salt solution into the same animals. The results of this test, based on 490 inoculations, are shown in Text-fig. 3. The data for the early stage placentas which were inactive are not included.



Text-Fig. 2. Effect of heterologous placenta extract on mouse carcinoma cells.

In the light of the above described results it would appear that the lack of inhibiting property encountered in the early cow, hog, and human placenta and term human placenta was probably due to the age of the organ rather than to the difference in species.[2] No doubt the

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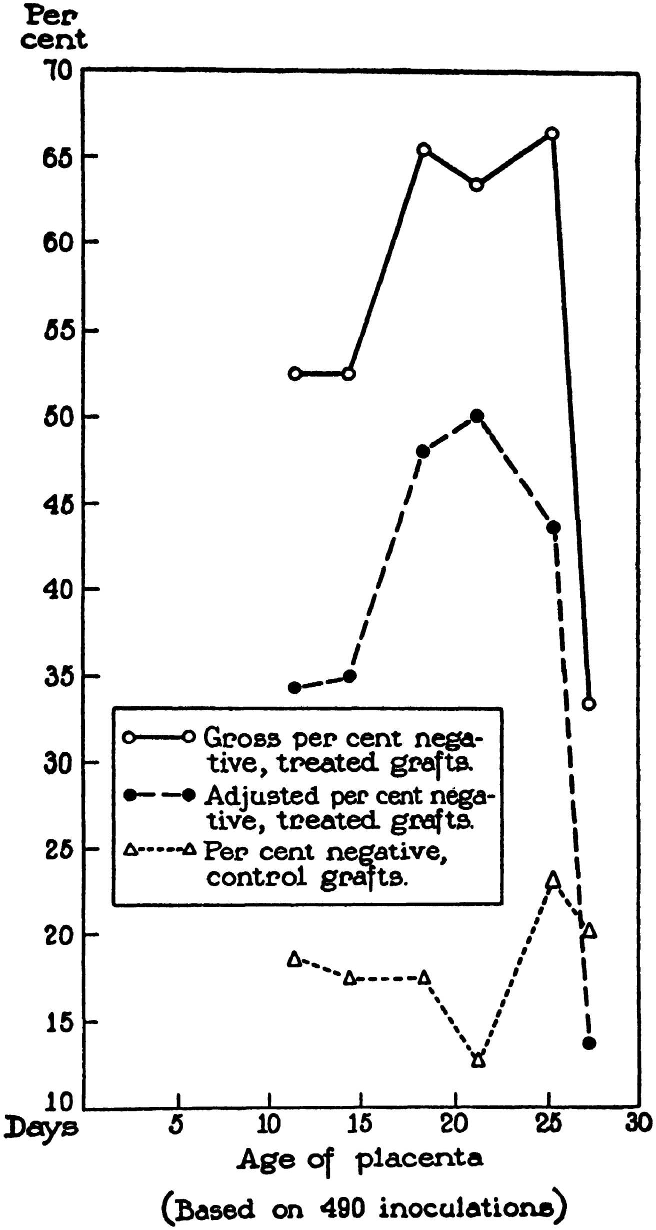
[2] Tests on cow placentas between the 7th and 8th month of pregnancy are in progress. The present indications are that appreciable amounts of the inhibiting factor are present.


Per cent
70
65
60
55
50
45
40
35
30
25
20
15
10
Days
5
10
15
20
25
30
Age of placenta
(Based on 490 inoculations)
○——○ Gross per cent negative, treated grafts.
●— —● Adjusted per cent negative, treated grafts.
△-----△ Per cent negative, control grafts.

TEXT-FIG. 3

curve as represented in Text-fig. 3 might be modified by an increased number of tests, but a sufficient number are available to establish the main points. There seems no immediate explanation in the sudden drop between the 26th and 28th days, 2 days before term.

DISCUSSION

This investigation was undertaken on the hypothesis that the balanced state and orderly growth of normal cells is maintained by the interaction of two forces, one which stimulates growth and the other which retards it. Under this hypothesis the break in the balance between the factors, either the loss of the retarding or the accumulation of the stimulating factor, would lead to uncontrolled growth. While the reported results demonstrate the presence of a retarding factor when the extracts of certain active tissues are tested against transplantable cancer—a fact which would seem to support the hypothesis—the question raised is far too complex to justify such a conclusion yet. Nor do we consider that there is sufficient evidence to show that the inhibitor from normal tissues is of the same order or acts in the same manner as the inhibitor isolated from certain fowl tumors. However, there are points of probable significance. The inhibitor from the fowl tumors could be demonstrated only in extracts from desiccated tumors, and the normal tissue factor is evident only in desiccates of placenta and embryo skin. With both materials their action is not species limited but appears to be tissue limited, the factor from chicken tumors acting only on sarcomas, and that from placenta and skin only on carcinomas. This specificity of action and the fact that the inhibitors are not demonstrable in extracts of fresh tissues are taken as evidence that they are not simply proteolytic enzymes or some other substance generally injurious to cells.

The explanation of the release of the inhibitor factor by desiccation of the tissues is not evident. It may be that in the tissues it is closely associated with the growth factor which neutralizes its action, and only becomes demonstrable when it is dissociated from the combination through desiccation.

Before the hypothesis suggested can be seriously entertained, the premise that normal cell balance is maintained by the interaction of two forces must be established. While there is a suggestion of some such mechanism in the work of Spemann (4) and others the principle is still far from being established.

SUMMARY

Extracts of desiccated embryo skin and placenta have been found to exert a definite retarding action on the growth of two transplantable carcinomas of mice, but they were without effect on sarcomas. In tests involving some 828 inoculations of the tumor cells and the extracts, complete suppression of growth occurred in from 55 to 71 per cent of instances, as compared with 21 to 27 per cent in the controls, and where growth was not completely suppressed some retardation was found in practically every instance. To judge from findings in rabbits the inhibitor is not demonstrable in the placenta until the beginning of the second third of pregnancy, reaches its maximum by the last third, but disappears about 2 or 3 days before term. Extracts of fresh placenta are without effect, and no very definite inhibition was noted in extracts of a variety of other desiccated or fresh tissues. The conclusions here reported are based on the results of over 3800 inoculations.

BIBLIOGRAPHY

1. Murphy, Jas. B., Helmer, O. M., Claude, A., and Sturm, E., *Science*, 1931, **73**, 266. Murphy, Jas. B., *Tr. Assn. Am. Physn.*, 1931, **46**, 182. Sittenfield, M. J., Johnson, A. S., and Jobling, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1931, **28**, 517. Gye, W. E., and Purdy, W. J., The cause of cancer, London, Cassell and Co., 461. Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1932, **56**, 103.
2. Murphy, Jas. B., and Sturm, E., *Science*, 1931, **74**, 180; *J. Exp. Med.*, 1932, **56**, 483.
3. Murphy, Jas. B., and Sturm, E., *Science*, 1932, **75**, 540 (preliminary note).
4. Spemann, H., *Naturwissenschaften*, 1933, **27**, 505.

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THE EFFECT OF A GROWTH-RETARDING FACTOR FROM NORMAL TISSUES ON SPONTANEOUS CANCER OF MICE*

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(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, June 21, 1934)

Extracts of placenta and embryo skin have a definite inhibiting action on the growth of transplantable cancers of mice (1). The absence of any effect of these materials on sarcomas, and the finding that the inhibiting factor isolated from certain fowl tumors acts on sarcomas and not on carcinomas, suggested a degree of tissue specificity for these factors. The attempt to establish a principle applying to cancer in general on evidence derived from the transplantable tumors only leaves something to be desired, for these tumors always represent cells foreign to the host that maintains them. Therefore, we have deemed it necessary to test the above mentioned factor on the spontaneous or natural cancers made up of malignant cells derived from the animal's own tissues (2).

The mammary tumors of mice have been studied because of the number available as by-products of the genetic studies, and because of the extensive data available on the natural history of these neoplasms. There is a surprising uniformity in the figures published by different investigators, using different strains of mice, on such points as the growth of autografts, local recurrence following surgical removal, spontaneous retrogression, and multiple foci of malignant change. In the present communication a report will be made on both the local and general effect upon these natural cancers of inhibiting materials that are known to act on transplanted tumors.

Materials

The tumor mice utilized came from three principal sources, and were from several strains. The largest number were from the general breeding room of

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* This investigation was carried out under the Rutherford Donation.

The Rockefeller Institute, where the stock of Swiss origin is inbred though not a pure line strain. Figures for the tumor rate, while known to be high, cannot be accurately determined by the available records. The second source was our genetic breeding room, where the mammary tumor strains are descendents of the original Abbe Lathrop stock. The third source, and the second largest, was the Roscoe Jackson Memorial Laboratory, and the animals came from several strains of high tumor incidence. The size of the tumors at the time that the mice were sent into the laboratory was rarely under 1.0 x 0.9 cm. and rarely over 1.8 x 1.5 cm. About 29 per cent of the animals had more than one primary tumor.

The extracts of mouse placenta and embryo skin were prepared in the manner described in earlier publications. The material, collected during the latter part of pregnancy, was minced, spread in a thin layer, and placed in a vacuum jar over sulfuric acid. After evacuation it was kept in the freezing box until desiccation was complete. The finely powdered material was thoroughly extracted with water (0.1 gm. to 1 cc.) and centrifuged. The supernatant fluid was used for the tests.

Effect of the Extracts on Local Postoperative Recurrence

The early invasion of the skin or deeper tissues by mammary cancer in mice is unusual. Probably owing to the looseness of the tissues, the tumors grow as more or less discrete masses in the subcutaneous tissue and can be easily separated. In spite of the apparent discreteness of the masses and the removal of all immediately adjacent normal tissue, local recurrence is not uncommon. The first tests were on the effect of the inhibiting extract on this manifestation.

Experiment.—The group was composed of 58 tumors removed and the wound bathed with embryo skin extract and 100 tumors removed and the operative field similarly treated with placenta extract. The operation consisted of a radical removal from the etherized animal of the tumor and adjacent tissue, under aseptic precautions. After the wound was sutured, from 0.05 to 0.2 cc. of the tumor extract, depending on the size of the wound, was injected into the area and then it was sealed with collodion to prevent leakage. In the controls collodion was used in the same way. There was rarely any evidence of infection and healing took place promptly. No further treatments were given and the animals were kept under close observation until their death. Diagnosis was made by sections of the original tumors and any nodules found at autopsy were removed and also sectioned. No animals were included as negative for local recurrence unless they survived over 5 weeks. The average length of life after operation was approximately 3 months for the group.

The results are shown in Table I, which for comparison includes our control series in which the tumors were removed without other treatment, two earlier series from this laboratory, and two groups reported by other investigators.

The recurrence rate following operative removal of mouse cancers reported by different investigators with different strains of mice is remarkably constant, the maximum variation between the groups being from 46.2 per cent to 54.0 per cent. The constancy of these figures gives even greater significance to the evidence of suppression of local recurrence by the two tissue extracts, particularly by placenta extract. While the figures for skin extract show a significant reduction over the expected recurrence rate it is doubtful if this represents the full potency of the material. The larger number of recurrences in this group took place in a lot of animals treated with a single preparation which was probably inactive; but this was not evident until too late to make further tests to establish the point. There seems no doubt that there is a definite reduction in local recurrence due to the treatment of the operative field with the tissue extracts.

TABLE I

Local Recurrence Following Operation

	No. operated	No. recurred	Percentage recurrence
Controls			
Murray*	48	23	47.9
Haaland†	174	96	54.0
Murphy and Morton‡	39	18	46.2
Nakahara§	50	26	52.0
Present series	144	69	47.9
Total controls	455	232	50.9
Placenta treated	100	6	6.0
Embryo skin treated	59	15	25.9

* Murray, J. A., *3rd Scient. Rep. Inv. Imp. Cancer Research Fund*, London, 1908, 69.

† Haaland, M., *4th Scient. Rep. Inv. Imp. Cancer Research Fund*, London, 1911, 1.

‡ Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, **22**, 800.

§ Nakahara, W., *J. Exp. Med.*, 1925, **41**, 347.

The Influence of the Tissue Extracts on the Fate of Autografts from Spontaneous Tumors

The autograft of a spontaneous tumor may be considered as an artificial metastasis and as such might be expected to grow in practically every instance. As a matter of fact, the percentage of takes is so high that it is probable that the failures are due to accidental loss of the graft through extrusion or destruction by local infection.

Experiments.—The experimental group for this test consisted of 111 mice with spontaneous mammary cancer. The primary tumors were removed surgically, and from the outer surface two large sized grafts were cut out. One of these was immersed for a few moments in one of the test fluids and the other in Ringer's solution for the same length of time. The two grafts were then reinoculated into opposite sides of the host of origin. The operation wound was also treated with the tissue extract. No further treatment was given to these animals and they were kept under observation for their duration of life. The diagnosis of the original tumors and the fates of the autografts were confirmed by microscopic sections.

The results are presented in two ways for better comparison. In Table II the figures are given for takes of the treated and untreated autografts in the same animals. Table III shows the figures for the untreated autografts compared with a larger number of controls in which autografts had been returned to animals without treatment of the animal or graft.

The figures for the growth of autografts collected in this laboratory and those reported by others are remarkably uniform, with the greatest variation between groups of 1.9 per cent. The failure of takes in 10.9 per cent and 18.5 per cent of the untreated grafts inoculated into animals which had received a treated graft and whose wound had been flushed with one of the tissue extracts, suggested that there is a general effect from the local injections. Here, as with the first experiments, it is doubtful if the figures for the effect of embryo skin extract give the correct idea of the full strength of this factor. Most of the positive grafts occurred in a lot of mice treated with an extract which we have reason to believe was inactive as stated in the foregoing experiment. The results cannot be explained on a possible interference between the double grafts. An analysis of an earlier unpublished study, in which 52 double autografts were made, showed that in 86.5 per cent both grew, and in only 7 per cent one grew and the other did not. The retardation of growth of tumors resulting from the treated graft, where inhibition was not complete, was very evident.

TABLE II

Effect of Embryo Skin and Placenta Extracts on Autografts of Tumors

	No. which grew	Per cent which grew	Per cent doubtful or no growth
46 tumors removed			
Autografts treated with embryo skin extract.	29	63.1	36.9
Autografts not treated..................	41	89.1	10.9
65 tumors removed			
Autografts treated with placenta extract....	21	32.3	67.7
Autografts not treated..................	53	81.5	18.5
64 tumors removed			
Autografts, no treatment of graft or wound...	62	96.9	3.1

	No.	Per cent
Autografts treated with embryo skin, negative or definitely smaller than control..................................	33	71.7
Autografts treated with placenta, negative or definitely smaller than control..................................	63	96.9

TABLE III

Growth of Autografts

	No. of autografts	No. which grew	Per cent which grew
Controls			
Haaland*..............................	62	60	96.9
Murphy and Morton*..................	29	28	96.6
Nakahara*............................	25	24	96.0
Additional Rockefeller Institute series......	202	192	95.0
Total controls..........................	318	304	95.6
Placenta treated........................	65	21	32.3
Embryo skin treated......................	46	28	60.9

* For references see Table I.

The Distal Effect of Inhibiting Factors on Tumors

The history of the established spontaneous cancers of mice, particularly the mammary tumors, has been studied by a number of investigators. The most complete data are those collected by Haaland (3) based on 353 tumors and those by Woglom (4) based on 2000 tumors. Haaland found five, or 1.4 per cent which retrogressed more or less completely, while Woglom had only 0.8 per cent which did not show a progressive course. Our figures would be close to Woglom's. We have not seen a well established tumor retrogress among the large number which have been followed in this laboratory. There was a suggestion of an effect on the control graft when the inhibiting extracts were injected into another part of the body, in the test just described. In the next experiments we have considered its possible action on an established primary tumor when injected at a distance.

Experiment.—In this group there were 142 primary tumors, 75 of which were treated by intraperitoneal injection of placenta extract and 67 with embryo skin extract. The injections of 1 cc. were made at weekly intervals, from six to fifteen injections, depending on the response of the tumor. A small piece was removed for diagnosis before the injections were started, in half the animals, and in the other half after the tumor had become stationary, or at autopsy. Otherwise the tumors were not interfered with. As there was no difference in the outcome of the treatments these animals were all grouped together. Weekly measurements were made and complete autopsies were performed at death. No tumors were included in the series unless the animal lived more than 5 weeks, except those dying as the result of growth of the tumors. The general health of the mice seemed unaffected by the treatment.

The results are shown in Table IV.

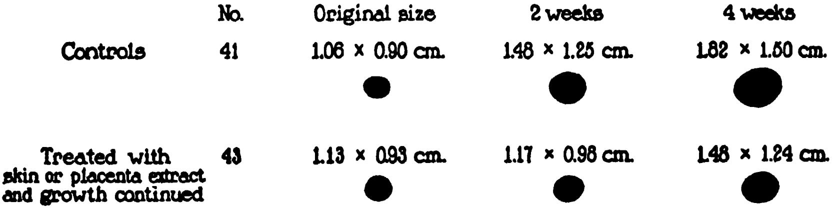
Among the two groups there were 45 tumors which continued to grow but apparently at a slower rate than in an untreated series. The average progress of 43 of these treated tumors in animals which lived for 4 weeks, compared with 41 of an untreated group is shown in Text-fig. 1. A comparison of this control group with a large number from earlier studies on growth rate shows this to be a fair average.

The growth-inhibiting action of the two tissue extracts is evidenced by the fact that 67 per cent and 69 per cent of the two groups treated showed no further growth of the tumors after the injections had been begun, and may be considered to have lived out their normal span of life, a number of the animals having survived over 6 months and two over a year. The tumors not completely stopped progressed at what appeared to be a slower rate than is usually shown by tumors of this type. The difference in effectiveness between the skin and placenta extracts is not marked here, for the use of an inactive preparation in one of a series of injections would not be so noticeable as in the first two experiments in which a single treatment was given.

TABLE IV

Effect of Embryo Skin and Placenta Extracts on Spontaneous Tumors

	No. of tumors	Continued growth	Stationary or slight retrogression	Marked retrogression	Complete absorption	Total showing inhibition
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
Treated with skin extract...........	67	32.8	28.4	14.9	23.9	67.2
Treated with placenta extract.......	75	30.7	21.3	26.7	21.3	69.3



TEXT-FIG. 1. The average growth rate of 43 spontaneous tumors in animals treated with placenta or embryo skin extracts, which continued growth after treatment, compared with the average growth rate of 41 untreated spontaneous tumors.

The number of complete regressions, 32 out of 142 tumors making up the series (a percentage of 22.5) is striking when compared with the fate of tumors in untreated mice in which spontaneous absorption occurs in less than 1 per cent. Tumors showing temporary retrogression followed by subsequent growth are not included among those classed as affected by the treatment. With untreated mice the rule is progressive growth until the death of the animal, but with considerable variation in the growth rate of individual tumors. Cessation of growth for any great length of time is unusual,[1] and retrogression to any marked degree is rarely seen. This experience is in agreement with Woglom's report, in which among 2000 mice with spontaneous tumors thirteen retrogressed and three fluctuated in growth rate, or remained stationary in size. Thus 99.2 per cent of 2000 untreated spontaneous cancers of mice pursued "their usual progressive course."

The Occurrence of New Primary Tumors in the Treated Groups

It is well known that mice not uncommonly have multiple primary foci of malignant change in the mammary tissue, the reported figures on this condition yielding an average of about 18 per cent. Haaland (5) collected the first systematic laboratory data and found that after the animal was sent into the laboratory with at least one established primary tumor, some 35 per cent developed additional new tumors before death. It is of interest to analyze the figures for the groups of mice in the above described experiments subjected to treatment with placenta or embryo skin extracts from the point of view of the new tumors developed.

Experiment.—All of the animals in the foregoing experiments were followed closely for their duration of life, and the appearance of new tumors or the finding of them at autopsy was recorded. They may be considered in two groups; one in which the tumors were removed, the operative field treated with one of the extracts, and in which each animal received an autograft which had been immersed in one of the extracts; in the second group the tumors were not removed and the mice received weekly intraperitoneal injections of one of the extracts, the total number varying from six to fifteen. For controls we have two earlier series followed in this laboratory, a recent series the direct control for the present experimental groups, and the figures published by Haaland. The collected data are given in Table V.

Here as with the other data collected from the studies of spontaneous cancer in mice, there is little variation between the figures collected by different investigators and from different strains. The incidence of new tumors in the untreated animals which had previously had one or more tumors, is 37.5 per cent, and the maximum spread between groups is from 32.0 to 43.0 per cent. Among the 210 treated mice the rate was 3.3 per cent, and in the 99 receiving several treatments only one new tumor developed, or a rate of about 1 per cent. These figures on the prevention of new foci of malignant change are as definite as those for the other points investigated. The study is being extended to cover the possibility of preventing the origin of a first tumor in mice of families normally showing a high tumor rate. The observation will include internal tumors as well as those of the mammary gland.

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[1] Slye believes that pregnancy retards the growth of spontaneous neoplasms in mice (Slye, M., *J. Cancer Research*, 1920, 5, 25), but as none of the mice in our series were pregnant this point need not be considered.

TABLE V

Development of New Tumors

	No. of tumor animals	No. which developed new tumors	Percentage which developed new tumors
Controls			
Haaland*	209	73	35.0
Nakahara*	50	16	32.0
Early Rockefeller Institute series	138	53	38.5
Present series	125	54	43.2
Total controls	522	196	37.5
Tumors removed			
One treatment skin extract	46	3	6.3
One treatment placenta extract	65	3	4.6
Tumors not removed			
Several injections skin extract	42	1	2.4
Several injections placenta extract	57	0	0.0
Total treated	210	7	3.3

* For references see Table I.

Histological Study

The tumors composing the above described experimental material represent the usual types of mammary gland neoplasm. Histologically those grafts or unoperated tumors which had been affected by the tissue extracts showed no striking change in their structure. The only prominent feature was the reduction or complete absence of mitotic figures in the tumors after treatment, compared with the biopsy sections. There was a suggestion of more differentiation in the treated tumors and some showed an increase in the connective tissue surrounding and invading the mass, but these differences were not sufficiently uniform to justify a conclusion as to their importance. No increase in cellular reaction was noted about the grafts, but a systematic investigation of the early changes was not made. A more complete study is in progress.

DISCUSSION

There are so many gaps in our evidence that it is untimely to discuss the bearing of these results on the hypothesis outlined in the foregoing paper (1), which has served as a basis for the investigation. There is no direct evidence as yet that the factor or substance in embryo skin and placenta extracts is related to the hypothetical control mechanism for normal cells. The lack of any reaction on the part of the host and the fact that the inhibitors are effective when the tumors are treated *in vitro* before reinoculation suggest that the action is a direct one on the cancer cells rather than a stimulation of the natural resistance mechanism of the body. Stimulation of the latter is the general interpretation accorded to the results obtained with generalized X-ray, dry heat, and fatty acids (6) which seem to represent an entirely different process from the action of the inhibitors. Our present impression is that the inhibitors affect directly the malignancy of the cell. However, any even tentative conclusion must await a more definite understanding of the control mechanism of normal cells.

The findings as concerns the various points investigated, prevention of postoperative recurrence, growth of autografts, the stoppage of further growth of well established cancer, followed in many instances by absorption of the tumor, and the prevention of new foci of malignancy, all indicate a very definite and pronounced effect of the extract of embryo skin and placenta on the growth and development of malignant cells. There were no direct controls for this series in which other tissue extracts were utilized, for such extracts had shown no inhibiting effect on transplanted cancer. It was judged more important to test the two materials which had proved effective with the transplantable tumors on the largest possible number of spontaneous cancers. We have records of unsuccessful attempts to influence the growth of spontaneous tumors by injection of homologous and heterologous serum, red blood cells, and a variety of other protein materials which would indicate that the present results are not due to some general non-specific reaction.[2]

SUMMARY

Extracts of desiccated homologous embryo skin and placenta decrease markedly the rate of postoperative local recurrence after the surgical removal of spontaneous cancer of mice. Autografts after a short period of contact with these extracts either failed to grow, or, in the majority of instances, their subsequent growth was definitely retarded. Intraperitoneal injection of the extracts was followed by cessation of growth of established tumors in more than two-thirds of the animals treated, and among these many of the tumors regressed and over 20 per cent were completely absorbed. Tumor mice treated with either extract rarely developed new malignant foci, though this happened frequently in untreated mice.

BIBLIOGRAPHY

1. Murphy, Jas. B., and Sturm, E., *Science*, 1932, **75**, 540; *J. Exp. Med.*, 1934, **60**, 293.
2. Preliminary notes: Murphy, Jas. B., and Sturm, E., *Science*, 1933, **77**, 631. Murphy, Jas. B., *Science*, 1933, **78**, 521.
3. Haaland, M., *4th Scient. Rep. Inv. Imp. Cancer Research Fund*, London, 1911, 1.
4. Woglom, W. H., *J. Cancer Research*, 1922, **7**, 379.
5. Haaland, M., *4th Scient. Rep. Inv. Imp. Cancer Research Fund*, London, 1911, 1.
6. Murphy, Jas. B., The lymphocyte in resistance to tissue grafting, malignant disease, and tuberculous infection. An experimental study, Monograph of The Rockefeller Institute for Medical Research, No. 21, New York, 1926.

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[2] This statement is based on an unpublished study of a large series of spontaneous tumors. No doubt other attempts of this sort have been made, and as negative results have not been reported.

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PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

IX. The Effect of Aqueous Extracts of Chicken Tumor I on Yeast Nucleic Acid*

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A component of the chromosomes and therefore intimately associated with the rate and rhythm of cell reproduction, nucleic acid is an interesting substrate for extracts of tumor material. The disintegration of nucleic acid by such extracts has been reported by several investigators including Somekawa (1), who observed that aqueous extracts of Chicken Tumor I caused a separation of purine derivatives from yeast nucleic acid, and Edlbacher and Kutscher (2), who claimed that mammalian tumors split phosphoric acid from thymus nucleic acid whereas extracts from Chicken Tumor I had little if any effect of this kind.

A review on nucleic acids by Levene (3) led to the belief that these isolated observations, reported without tests for biological potency, might be correlated and extended, and to the opinion that an important structural characteristic of nucleic acid had been ignored; namely, that the arrangement of nitrogenous, sugar, and phosphoric acid groups is such that the ester bonds between component nucleotides must be broken to release more than one-quarter of its phosphoric acid and that they may be broken without release of inorganic phosphorus or of nitrogenous bases. This division of nucleic acid into its four nucleotides when the ester bonds are broken by enzyme action may be called polynucleotidase activity.[1]

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* This investigation was carried on under the Rutherford Donation.

[1] Levene and Dillon (4) use the same term for the release of an arbitrary amount of inorganic phosphorus in unit time from nucleic acid by an enzyme from intestinal juice.

The polynucleotidase and phosphatase activities of aqueous ex of Chicken Tumor I are reported in this paper with evidence d strating a relationship between two systems, polynucleotidase ac and associated inhibiting material, and tumor-inducing agent and ciated inhibiting material.

Methods

As defined in the foregoing, polynucleotidase activity is the division of acid into its four component nucleotides and therefore the measurement activity depends on the separation of nucleic acid from nucleotides. This tion was accomplished by precipitating nucleic acid at its iso-electric poi less than 2.0, with a solution of 1.25 gm. uranyl chloride in 10 per cent tr acetic acid. This mixture yields a supernatant solution of nucleotides a complex derivatives of nucleic acid which can be separated from the precipi intact nucleic acid by spinning the mixture in a centrifuge (9).

As a source of such enzyme activity various products of Chicken Tumor tested. These products were Berkefeld filtrates of fresh tumor material, ac extracts of desiccated tumor material, and the supernatant fluid obtain treating the aqueous extract with aluminum hydroxide Type C prepa described by Willstätter and Kraut (5). The details of preparation of products may be found in the papers of Murphy and his associates (6) extracts used in the following experiments were prepared by extracting 1 gm tumor powder with 60 cc. distilled water, separating the insoluble matt spinning in a centrifuge at 1200 R.P.M. for 10 minutes, and decanting the natant soluble substances. The aluminum supernatant fluids were prepar shaking a volume of aqueous extract with the gel from an equal volume of a num hydroxide Type C, spinning the mixture in a centrifuge at 1200 R.P.M. minutes, and decanting the supernatant fluid. This supernatant fluid is re to as *Sa*. The concentration of chicken tumor substance in solution was mined by drying 2 cc. of solution under reduced pressure in a desiccator conta concentrated sulfuric acid until the weight of dried material was constant.

The disintegration of yeast nucleic acid was observed in mixtures contain volume, 1 to 5 cc., of chicken tumor product, 3 volumes of yeast nucleic ac 0.15 M buffer solution, and 1 volume of an aqueous solution of phenol. mixture is called the digestion mixture throughout the paper. The buffers for pH's of 5.0 to 7.0 were the sodium hydroxide-sodium citrate mixtures pre according to Clark (7), and for pH's of 7.0 to 9.0 the sodium veronal-hydroc acid mixtures prepared according to Michaelis (8). The final concentrati nucleic acid was 1 to 4 per cent and of phenol was no greater than 0.3 per The digestion mixtures, unboiled and boiled, were incubated in tubes with ru stoppers at 37.5°C. until aliquots taken at regular intervals indicated that n mum disintegration of nucleic acid had been attained. Each aliquot was examined bacteriologically by smear and by subculture on agar because the final concentration of phenol was of low antiseptic power particularly for members of the aerobic, spore-bearing group of bacilli whose intensive disintegration of yeast nucleic acid is to be reported elsewhere with a more detailed description of the chemical methods (9).

The products of disintegration of nucleic acid were separated from intact nucleic acid by the following procedure. 4 cc. samples of the digestion mixtures were pipetted into centrifuge tubes of 50 cc. capacity and to each sample was added 11 cc. distilled water and 10 cc. of the precipitating reagent. The mixture was shaken vigorously for a few seconds and stirred thoroughly twice during a 10 minute interval. The white flocculent precipitate was separated from the yellow supernatant fluid by spinning the mixture in a centrifuge at 900 R.P.M. for 3 minutes. Then the supernatant fluid was decanted into a 50 cc. volumetric flask, and the precipitate was broken up with 10 cc. of precipitating solution previously diluted to 2/5 concentration with distilled water. Separation of the precipitate and supernatant was repeated twice as described and the three supernatants pooled and made up to 50 cc. with dilute precipitating reagent. The precipitate was dissolved in 25 cc. of N sodium carbonate.

In this way two solutions were made available for analysis of nitrogen and of phosphorus. Total nitrogen was determined by the micro-Kjeldahl method of Van Slyke (10); inorganic phosphorus was determined by the colorimetric method of Leiboff (11); and total phosphorus was determined by a modification of the method of Leiboff (9). From these analyses of the two solutions the polynucleotidase and phosphatase activities were calculated by the use of appropriate factors.

The estimations of enzyme activity were based on the structural formula of nucleic acid reported by Levene (3). Polynucleotidase activity was calculated, in terms of milligrams of nucleic acid disintegrated, by multiplying by 6.13 both the increase of total nitrogen soluble in the uranyl chloride supernatant fluid and the decrease of total nitrogen in the uranyl chloride precipitate, and by multiplying by 10.38 both the increase of total phosphorus in the uranyl chloride supernatant and the decrease of total phosphorus in the uranyl chloride precipitate. Similarly phosphatase activity was calculated, in terms of milligrams of nucleic acid disintegrated to release inorganic phosphorus, by multiplying by 10.38 the inorganic phosphorus soluble in the uranyl chloride supernatant fluid. The factor 6.13 is the ratio of the molecular weight of yeast nucleic acid to the weight of 15 atoms of nitrogen; *i.e.*, 1287.5/210. The factor 10.38 is the ratio of the molecular weight of yeast nucleic acid to the weight of 4 atoms of phosphorus; *i.e.*, 1287.5/124.1.

Except where stated each observation in the tables showing results is the difference of analysis of boiled digestion mixture from that of unboiled digestion mixture.

RESULTS

Fifty aqueous extracts from different Chicken Tumor I powders have been investigated for hydrolysis of yeast nucleic acid: forty-four caused a disintegration of nucleic acid as considerable as that of chicken liver, kidney, spleen, and much more considerable than that of muscle. The other six extracts caused little or no disintegration of nucleic acid for reasons possibly associated with their pathological character; *i.e.*, the induction or inhibition of tumor growth.

Variations in the tumor-inducing power of different Chicken Tumor I powders have been noted by Murphy and Sturm (12), who classify the pathological activity of such powders as *active*, *neutral*, *inhibiting*: an *active* powder yields an aqueous extract which will induce an easily measurable tumor in 10 to 14 days after the extract is injected intracutaneously into the breasts of barred Plymouth Rock hens; an *inhibiting* powder, usually from a slow growing tumor, yields an aqueous extract which, when mixed with an active extract, prevents completely the appearance of a tumor for at least 5 weeks after injection; and a *neutral* powder, usually one which has been kept more than a year, neither induces nor inhibits tumor growth. The aluminum supernatant fluid when obtained from an active extract is called *active Sa*, when obtained from a neutral extract is called *neutral Sa*, and when obtained from an inhibiting extract is called *inhibitor Sa*. The aluminum supernatant fluids are either active or neutral pathologically but never inhibiting because the aluminum hydroxide gel adsorbs the inhibiting material to a greater degree than it does the tumor-inducing agent.

The foregoing definitions have been made to avoid confusion between enzyme and biological activities: in all cases the pathological term is given to the source of enzyme activity. Further aids to clear understanding of the tables are the summaries at the head of each table which give the final concentration of substrate, source of enzyme activity, and of antiseptic, and which give the pH, time of incubation at 37.5°C., and the results of subculture on agar slopes. Finally, under the heading "Nucleic acid disintegrated" the number (1) refers to nucleic acid calculated from the total nitrogen in the uranyl chloride supernatant fluid, the number (2) refers to nucleic acid calculated from the total phosphorus therein, the number (3) refers to nucleic acid calculated from the total nitrogen in the uranyl chloride precipitate, and the number (4) refers to the nucleic acid calculated from the total phosphorus therein.

Polynucleotidase Activity of Tumor-Inducing Extracts of Chicken Tumor I.—The polynucleotidase activity of active extracts of Chicken Tumor I caused a maximum disintegration of 44 per cent of yeast nucleic acid in 1 week at 37.5°C. at pH of 6.5. The enzyme activity (as well as the tumor-inducing activity) was destroyed by heating the extracts at temperatures above 55°C. for 30 minutes. These statements are substantiated in Table I.

TABLE I

Polynucleotidase Activity of Aqueous Extracts of Chicken Tumor I Powders

Nucleic acid, 1 per cent.
Chicken Tumor I, 0.22 per cent.
Phenol, 0.22 per cent.

pH, 6.5.
Time of incubation, 1 week.
Subcultures on agar, negative.

Source of enzyme activity	(*A*) Nucleic acid disintegrated		(*B*) Nucleic acid left intact		(*C*) Hydrolysis $C = \frac{A}{A+B} \times 100$
	(1)	(2)	(3)	(4)	
	mg.	*mg.*	*mg.*	*mg.*	*per cent*
Active extract	90.72	90.83	113.96	115.74	44
Active extract	90.11	90.82	115.06	116.77	44
Boiled extract	6.13	2.07	198.61	204.49	3
Boiled extract	5.52	2.07	199.22	203.45	3

TABLE II

Polynucleotidase Activities of Extracts of Chicken Tissues

Nucleic acid, 3.75 per cent.
Phenol, 0.1 per cent.
pH, the optimum for each extract.
Time of incubation, 1 week.

Dry weight per cc. of extract:

Liver, kidney	18 mg.
Spleen	16 mg.
Chicken Tumor I	11 mg.
Muscle	8 mg.

Source of enzyme activity	Optimum pH	Nucleic acid disintegrated (2)	Nucleic acid disintegrated per mg. dry weight of extract
		mg.	*mg.*
Liver	8.8	62.80	3.49
Kidney	8.8	52.42	2.91
Chicken Tumor I	7.5	45.15	4.10
Spleen	8.8	43.08	2.70
Muscle	7.0	5.19	0.65

Compared with those normal tissues of high polynucleotidase capacity Chicken Tumor I has an activity between that of kidney and that of spleen and much greater than that of muscle. In this experiment all extracts of normal tissues were prepared as described for extracts of Chicken Tumor I and the dry weight per cubic centimeter of extract determined. The polynucleotidase activities of these extracts are given in Table II.

Consideration of Table II reveals that the unit activity per milligram dry weight was greater than that of any normal tissue investigated.

Differences between Polynucleotidase and Phosphatase Activities of Active Extracts of Chicken Tumor I.—The polynucleotidase and phosphatase activities of extracts of Chicken Tumor I can be distinguished by the rate of hydrolysis, the effect of hydrogen ion concentration, and the effect of low oxygen tension.

TABLE III

Rates of Activity of the Polynucleotidase and Phosphatase of Chicken Tumor I

Nucleic acid, 1 per cent.
Chicken Tumor I, 0.22 per cent.
Phenol, 0.22 per cent.

Time of incubation, 4 weeks.
pH, 6.5.
Subcultures of agar, negative.

Time of incubation	(*A*) Nucleic acid disintegrated		(*B*) Nucleic acid disintegrated to release inorganic phosphorus	(*C*) Ratio of polynucleotidase to phosphatase $C = A/B$
	(1)	(2)		
days	*mg.*	*mg.*	*mg.*	
0	5.50	4.88	2.07	2.5
1	26.36	26.16	6.54	4.0
2	44.75	43.70	12.14	3.6
5	76.13	75.77	24.39	3.1
7	90.72	90.82	32.59	2.8
14	91.95	93.42	41.52	2.3
21	93.79	94.15	48.89	1.9
28	93.79	94.15	49.30	1.9

The rate of hydrolysis for each enzyme activity at pH 6.5 is given in Table III, which shows that the polynucleotidase starts faster and ends sooner than the phosphatase, and that no more than one-third of the disintegrated nucleic acid released inorganic phosphorus.

The optimum pH for polynucleotidase activity is about 7.5, whereas it is about 6.5 for phosphatase activity, as shown in Table IV.

An environment of low oxygen tension was produced in a glass jar, containing the digestion mixtures in tubes without stoppers, after withdrawal of air, by restoring atmospheric pressure with hydrogen. In this environment the phosphatase activity was twice that in air whereas the polynucleotidase activity was the same as in air, as shown in Table V.

Phosphatase Activity of Tumor-Inducing Extracts of Chicken Tumor I.—The phosphatase activity of active extracts of Chicken Tumor I caused a maximum disintegration of 15 per cent of the nucleic acid in the digestion mixture at pH of 6.5 in 3 weeks at 37.5°C. The inorganic phosphorus released from nucleic acid may have come from it directly or indirectly through nucleotides or through phosphoric acid ester of ribose as the immediate substrate. This alternative is preferred because there is a definite lag of the phosphatase behind the polynucleotidase activity. Therefore, the phosphatase activity is calculated in terms of percentage hydrolysis of disintegrated nucleic acid. So defined, the phosphatase activity of Chicken Tumor I is much less

TABLE IV

Effect of Hydrogen Ion Concentration on the Polynucleotidase and Phosphatase Activities of Chicken Tumor I

Nucleic acid, 2 per cent.	Time of incubation, 3 weeks.
Chicken Tumor I, 0.37 per cent.	pH, as shown.
Phenol, 0.1 per cent.	Subcultures on agar, negative.

pH	(*A*) Nucleic acid disintegrated		(*B*) Nucleic acid disintegrated to release inorganic phosphorus	(*C*) Ratio of polynucleotidase to phosphatase $C = A/B$
	(1)	(2)		
	mg.	*mg.*	*mg.*	
5.0	144.0	144.6	79.41	1.80
5.5	150.0	149.7	80.96	1.85
6.0	153.2	154.5	90.82	1.70
6.5	159.0	157.5	99.13	1.59
7.0		162.4	78.37	2.07
7.5		171.2	39.44	4.35
8.0		160.7	12.46	12.89
8.5		152.4	8.30	18.40
9.0		142.1	10.38	13.69

intense than that of any extract of normal tissue investigated and it has an optimum pH different from that of the polynucleotidase. These statements are substantiated in Table VI.

TABLE V

Polynucleotidase and Phosphatase Activities of Chicken Tumor I Extracts in an Environment of Air and of Hydrogen

Nucleic acid, 3.75 per cent.
Chicken Tumor I, 0.30 per cent.
Phenol, 0.12 per cent.

Time of incubation, 1 week.
pH, 7.3.
Subcultures on agar, negative.

Atmospheric environment	(*A*) Nucleic acid disintegrated (2)	(*B*) Nucleic acid disintegrated to release inorganic phosphorus	(*C*) Polynucleotidase activity $C = A/150$	(*D*) Phosphatase activity $D = B/150$
	mg.	*mg.*	*per cent*	*per cent*
Hydrogen	71.8	22.0	47.9	14.4
Hydrogen	71.8	21.8	47.9	14.2
Hydrogen	71.8	22.0	47.9	14.2
Air	72.7	10.0	48.5	6.5
Air	71.8	11.2	47.9	7.3
Air	70.9	10.6	47.3	6.9

TABLE VI

Phosphatase Activities of Extracts of Chicken Tissues

Nucleic acid, 3.75 per cent.
Phenol, 0.1 per cent.

Time of incubation, 3 weeks.
pH, the optimum for each extract.

	Optimum pH	(*A*) Nucleic acid disintegrated (2)	(*B*) Nucleic acid disintegrated to release inorganic phosphorus	(*C*) Phosphatase activity $A = \frac{A}{B} \times 100$
		mg.	*mg.*	*per cent*
Liver	8.8	62.80	38.57	61
Kidney	8.8	52.42	32.49	62
Chicken Tumor I	6.5	40.16	13.39	33
Spleen	8.8	43.08	24.46	57
Muscle	7.0	5.19	4.15	80

Polynucleotidase and Phosphatase Activities of Sa from Active Extracts of Chicken Tumor I.—Adsorption of material from aqueous extracts by aluminum hydroxide Type C in most cases increases the absolute intensity of polynucleotidase activity and always (twenty-five tests) increases the unit activity per milligram dry weight of Chicken Tumor I in solution. On the other hand, the absolute intensity of the phosphatase activity in all cases is decreased, and the unit activity increased though not to as great a degree as the polynucleotidase. The effect of adsorption with aluminum hydroxide Type C on the enzyme activities of the extracts is shown in Table VII. The frequent increase of total activity caused by treating the extract with aluminum hydroxide gel suggested that some inhibiting substance had been removed by adsorption. Similar experiments by Murphy and associates (12) had disclosed the existence of material which inhibited the tumor agent, and for this reason the suggestive relationship between the two systems, polynucleotidase and associated inhibiting substance, and tumor agent and associated inhibiting substance, was tested more definitely.

TABLE VII

Effect of Adsorption with Aluminum Hydroxide Type C on the Polynucleotidase and Phosphatase Activities of Extracts of Chicken Tumor I

Nucleic acid, 1.87 per cent.
Chicken Tumor I in extract, 11.1 mg. per cc.
Chicken Tumor I in Sa, 1.7 mg. per cc.
Phenol, 0.1 per cent.

Time of incubation, 3 weeks.
pH, 6.5.
Chicken Tumor I in digestion mixture:
with extract..... 33.3 mg.
with Sa......... 5.1 mg.

Source of enzyme activity	(*A*) Nucleic acid disintegrated (2)	(*B*) Nucleic acid disintegrated to release inorganic phosphorus	(*C*) Polynucleotidase activity per mg. Chicken Tumor I $C = A/33.3$ or $A/5.1$	(*D*) Phosphatase activity per mg. Chicken Tumor I $D = B/33.3$ or $B/5.1$
	mg.	*mg.*	*mg.*	*mg.*
Extract	73.14	46.81	2.2	1.4
Extract	72.77	44.84	2.2	1.3
Sa	81.26	24.60	15.9	4.8
Sa	79.96	24.29	15.7	4.8

Polynucleotidase Activities of Sa's of Graded Tumor-Inducing Power. —Because the polynucleotidase activity caused a maximum disintegration of nucleic acid in 1 week and because the tumors reach a measurable size in 10 to 14 days it was possible to predict the relative size of tumors induced by Sa's of active, neutral, and inhibiting extracts. Within the error associated with the use of only two dimensions as accurate indices of the size of the tumors there was a rough proportionality between the polynucleotidase activity and the size of the tumors induced by these aluminum supernatant fluids. This proportionality is demonstrated in Table VIII, and was repeated in the only other experiment of this kind done.

TABLE VIII

Polynucleotidase Activities of Sa's of Graded Tumor-Inducing Power

Nucleic acid, 1.8 per cent. Chicken Tumor I in active Sa, 1.7 mg. per cc.
Phenol, 0.1 per cent. Chicken Tumor I in neutral Sa, 1.6 mg. per cc.
Time of incubation, 3 weeks. Chicken Tumor I in inhibitor Sa, 0.9 mg. per cc.
pH, 6.5. Subcultures on agar, negative.

Source of enzyme activity	(*A*) Nucleic acid disintegrated (2)	(*B*) Chicken Tumor I in digestion mixtures	(*C*) Nucleic acid disintegrated per mg. Chicken Tumor I *C* = *A*/*B*	(*D*) Maximum and minimum size of tumors (6 injections for each Sa)
	mg.	*mg.*	*mg.*	*cm.*
Active Sa	81.26	5.1	15.9	2.8 x 2.0 to 2.6 x 2.1
Active Sa	79.96	5.1	15.7	
Neutral Sa	17.85	4.8	3.7	0.7 x 0.5 to 0.4 x 0.3
Neutral Sa	17.27	4.8	3.6	
Inhibitor Sa	3.31	2.7	1.2	No growth
Inhibitor Sa	3.99	2.7	1.5	

Polynucleotidase Activity of an Active Sa When Mixed with Extracts of Graded Tumor-Inhibiting Power.—Extracts of graded tumor-inhibiing power were prepared from active, neutral, and inhibiting powers in the usual way. Each extract was heated to 55°C. for 30 minutes to destroy any polynucleotidase and tumor-inducing activity, cooled to room temperature, and mixed with an equal volume of an active Sa.

6 cc. of this mixture was tested for polynucleotidase activity with 14 cc. of 2.5 per cent yeast nucleic acid in buffer at pH of 6.5, and 0.4 cc. was injected intracutaneously into the breasts of barred Plymouth Rock hens. Both experiments performed in this way demonstrated, as in Table IX, a rough proportionality between inhibition of the tumor agent and inhibition of the enzyme activity.

TABLE IX

Polynucleotidase Activity of an Active Sa When Mixed with Equal Volumes of Extracts of Graded Tumor-Inducing Power

Nucleic acid, 1.75 per cent.
Phenol, 0.22 per cent.
pH, 6.5.

Time of incubation, 3 weeks.
Subcultures on agar, negative.

Pathological character of extracts mixed with active Sa	(*A*) Nucleic acid disintegrated. Extract heated to 55°C. (2)	(*B*) Nucleic acid disintegrated. Extract boiled (2)	(*C*) Inhibition of polynucleotidase activity $C = \frac{B - A}{B} \times 100$	Maximum and minimum size of tumors (6 injections for each mixture of extract and Sa)
	mg.	*mg.*	*per cent*	*cm.*
Active extract	24.13	25.09	3.8	1.7 x 1.6 to 1.5 x 1.5
Neutral extract	14.82	24.64	40.0	0.9 x 0.8 to 0.8 x 0.8
Inhibiting extract	5.75	25.62	77.6	No growth
Water	25.70	25.70	0	1.8 x 1.6 to 1.6 x 1.6

DISCUSSION

The results reported in this paper confirm the observations of Edlbacher and Kutscher that extracts of Chicken Tumor I have very little phosphatase activity at pH's greater than 8.0 and are consistent with the report of Somekawa that these extracts cause a separation of purine substance from yeast nucleic acid more considerable than that of muscle. All these results may be correlated by a tentative explanation that under the influence of enzymes in Chicken Tumor I yeast nucleic acid undergoes a progressive disintegration through the stage of nucleotides to phosphoric acid ester of ribose and free nitrogenous bases.

The pathological significance of the polynucleotidase in Chicken Tumor I products cannot be stated definitely, though the rough proportionality between the enzyme system and the pathological system, the occurrence of the optimum pH in the physiologically important zone of pH 7.0 to 7.8, and the similarity of the minimal lethal temperature lead to the opinion that the relationship is not haphazard.

SUMMARY

1. Aqueous extracts of Chicken Tumor I cause a disintegration of yeast nucleic acid.
2. Two enzyme activities, a polynucleotidase and a phosphatase, are demonstrated.
3. These activities are distinguished by different reactions to an environment of hydrogen, different optima of hydrogen ion concentration, and different rates of activity.
4. Adsorption with aluminum hydroxide Type C removes substances inhibiting the polynucleotidase activity.
5. There is a rough proportionality between the polynucleotidase activity and the tumor-inducing power of different fractions of extracts of Chicken Tumor I.
6. There is a rough proportionality between the inhibition of polynucleotidase activity and the inhibition of the tumor agent.
7. The polynucleotidase activity of Chicken Tumor I is as great as that of spleen, slightly less than that of kidney and liver, but much greater than that of muscle. There are also qualitative differences.

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BIBLIOGRAPHY

1. Somekawa, E., *Sc. Papers Inst. Phys. Chem. Research (Tokyo)*, 1932, **18**, 23.
2. Edlbacher, E., and Kutscher, W., *Z. physiol. Chem.*, 1931, **199**, 1200.
3. Levene, P. A., Nucleic acids, New York, The Chemical Catalog Co., 1931, 274, 284.

4. Levene, P. A., and Dillon, R. T., *J. Biol. Chem.*, 1932, **96**, 461.
5. Willstätter, R., and Kraut, H., *Ber. chem. Ges.*, 1923, **56**, 149, 1117.
6. Murphy, Jas. B., Sturm, E., Claude, A., and Helmer, O. M., *J. Exp. Med.*, 1932, **56**, 91.
7. Clark, W. M., The determination of hydrogen ions, Baltimore, The Williams & Wilkins Co., 1st edition, 1920, 182.
8. Michaelis, L., *J. Biol. Chem.*, 1930, **87**, 33.
9. MacFadyen, D. A., *J. Biol. Chem.*, in press.
10. Van Slyke, D. D., *J. Biol. Chem.*, 1927, **71**, 235.
11. Leiboff, S. L., *J. Lab. and Clin. Med.*, 1931, **16**, 495.
12. Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1932, **56**, 107.

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THE NUCLEASE ACTIVITY OF BACILLUS SUBTILIS

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Nuclease activity was dissociated from proteolytic activity when Sachs confirmed the observations of Milroy that an agent which attacked nucleic acid to liberate inorganic phosphoric acid could not liquefy gelatin and that proteolytic enzymes liquefied gelatin but could not attack nucleic acid (1). Henceforth the rate of separation of inorganic phosphoric acid from nucleic acid became the exclusive index of nuclease activity (2–4). Activity so defined was shown by a variety of microorganisms, such as yeast (5), *Penicillium glaucum*, and *Aspergillus niger* (6), and by *Bacillus coli*, *Bacillus proteus*, and staphylococcus (7).

Convincing evidence of bacterial disintegration of nucleic acid other than the slow separation of phosphoric acid has been lacking despite the discovery of a series of enzymes which can attack the different components of nucleic acid: thus there are polynucleotidases which subdivide nucleic acid to nucleotides,[1] nucleotidases which split mononucleotides to phosphoric acid and nucleosides, nucleosidases which disjoin the sugar and nitrogenous base, and phosphatases which split phosphoric acid esters of ribose to free acid and free sugar ((8) pp. 311–316). Furthermore, the accepted structural formula of yeast nucleic acid, that elucidated by Levene and Bass ((8) p. 274, 286) indicates that the separation of more than one-quarter of the phosphoric acid in nucleic acid requires the breaking of the ester bonds between its component nucleotides. It is apparent also that these ester bonds may be broken without the separation of phosphoric acid from any of the four nucleotides and that the study of nuclease activity must encompass more than the determination of inorganic phosphoric acid.

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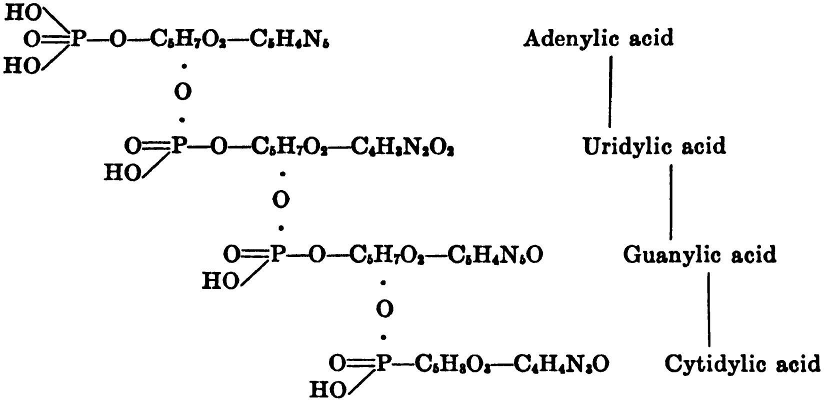
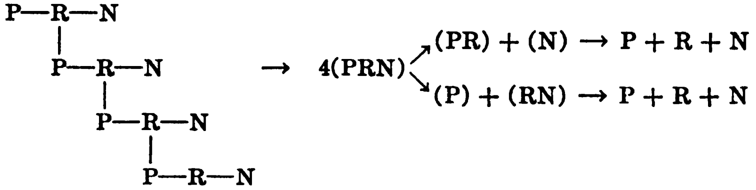
[1] Levene and Dillon use the term polynucleotidase activity to describe the liberation of an arbitrary amount of phosphoric acid from nucleic acid in unit time by an enzyme from intestinal juice (3).

Substantiation for the foregoing argument is to be found by inspection of the structural formula of yeast nucleic acid and of the convenient condensed forms presented with the accompanying scheme representing the disintegration of nucleic acid. In this scheme P represents phosphoric acid, R represents ribose, and N represents nitrogenous base.





The rate and process of disintegration of yeast nucleic acid may be deduced from the nitrogen and phosphorus in three fractions obtained by the methods described in this report. When these methods were applied to cultures of bacteria in solutions of yeast nucleic acid, the results revealed the rapid, intensive decomposition of nucleic acid by *Bacillus mesentericus*, *Bacillus megatherium*, and *Bacillus subtilis*.

Methods

The methods used in the following experiments were extensions of methods of previous investigators. Levene and Bass had used neutral lead acetate to separate nucleosides from phosphoric derivatives of nucleic acid ((8) p. 167), and Buell and Perkins had used uranyl nitrate to precipitate quantitatively the purine nucleotides from tungstic acid filtrates of defibrinated blood (9). I found that yeast nucleic acid was precipitated completely from dilute solutions of uranyl chloride in trichloroacetic acid at pH 1.5 to 2.0, and that under similar conditions the purine nucleotides and cytidylic acid were precipitated only in the range of pH 3.0 to 7.0, whereas uridylic acid and the less complex derivatives of nucleic acid were soluble at any pH tried from 1.5 to 9.0. The complete precipitation of nucleic acid is demonstrated in Table II, which shows that 94 per cent of the material in the control solutions was precipitated and that the remaining 6 per cent represented nucleic acid which had been decomposed before the culture mixtures were prepared; the phosphorus-containing substance not precipitated was found to be present in the same amount whether the supernatant solution was analyzed after or without digestion with strong acid.

The following detailed description includes the procedure for the preparation of cultures of bacteria in solutions of yeast nucleic acid and embraces the methods of analysis of these culture mixtures.

Preparation of Culture Mixtures—Agar slopes were seeded with bacteria and incubated for 18 to 24 hours at 37.5°. A suspension of living bacteria was prepared by emulsification of these colonies with 5 cc. of distilled water. The substrate was prepared by dissolving 4 gm. of sodium nucleinate in 100 cc. of buffer solution at a desired pH. 4 cc. samples of this solution were distributed in culture tubes which were stoppered with cotton and autoclaved. 1 cc. of bacterial suspension or 1 cc. of distilled water, as control, was mixed with 4 cc. of autoclaved substrate, in tubes stoppered with sterile rubber caps, and then incubated at 37.5° for 48 hours.

For a range of pH 7.0 to 9.6 the buffers used, in concentration of 0.1 M, were the sodium veronal-hydrochloric acid mixtures prepared as described by Michaelis (10), and for a range of pH 5.0 to 7.0 the buffers used, in concentration of 0.2 M, were the sodium citrate-sodium hydroxide mixtures, according to Clark (11).

Method for Separation of Nucleic Acid from Culture Mixtures—The contents, 5 cc., of each culture tube were washed with water into large centrifuge tubes of 50 cc. capacity and the volume made to 15 cc. with distilled water and then to 25 cc. with the acid-precipitating reagent. A flocculent precipitate which began to settle in a few seconds was stirred thoroughly twice during a 10 minute interval. By spinning the mixture in a centrifuge at 900 R.P.M. for 3 minutes it yielded a supernatant fluid, which was then decanted into a 50 cc. volumetric flask, and a precipitate, which was broken up by stirring with 10 cc. of dilute reagent and again separated by spinning in a centrifuge at 900 R.P.M. for 3 minutes. This operation was repeated once more to redissolve any adherent material less complex than nucleic acid. The supernatant fluids were pooled and made up to 50 cc. with dilute reagent, and the precipitate was dissolved in 25 cc. of 0.5 N sodium carbonate.

The acid-precipitating reagent was a solution of 1.25 gm. of uranyl chloride in 100 cc. of 10 per cent trichloroacetic acid. The dilute reagent was prepared by diluting this to 2½ volumes with distilled water. Neither of the buffer systems used, in concentrations up to 0.2 M, was precipitated by these reagents.

Method for Precipitation of Nucleotides from Uranyl Chloride Supernatant Liquids—10 cc. of uranyl chloride supernatant fluid were pipetted into a centrifuge tube and neutralized by adding about 2 cc. of N sodium carbonate, drop by drop, until the pH of the mixture was 6.8 to phenol red (as an outside indicator). During this adjustment a precipitate forms, beginning at pH 3.0, reaching a maximum size at about pH 4.3, and dissolving in greater part at pH 6.8. Drops of a solution of 10 per cent neutral lead acetate were added to this slightly acid mixture until no more precipitate could be produced by adding 10 drops more to the supernatant fluid previously cleared by spinning in a centrifuge at 900 R.P.M. for 3 minutes. Hydrogen sulfide was passed through the supernatant fluid to precipitate any excess reagent, which was then separated by spinning the mixture in a centrifuge at 1200 R.P.M. for 5 minutes. This supernatant fluid was aerated to remove excess hydrogen sulfide and then decanted into a volumetric flask where the volume was made up to 25 cc. with distilled water. The sodium veronal-hydrochloric acid buffers are completely precipitated by this procedure.

Methods of Analysis for Nitrogen and Phosphorus—The foregoing methods give three fractions of the culture mixtures, the carbonate solution of the uranyl chloride precipitate (Fraction 1), the uranyl chloride supernatant fluid (Fraction 2), and the supernatant fluid after treatment with neutral lead acetate (Fraction 3). The unfractionated culture mixture is labeled Fraction 4.

Small samples, 1 to 5 cc., were taken from each of the three fractions and analyzed for *total nitrogen* by means of the micro-Kjeldahl method of Van Slyke (12). *Inorganic phosphorus* (*i.e.* phosphoric acid not bound to organic substance), in 2 to 5 cc. of Fraction 2, was determined either by Tisdall's (13) or Leiboff's (14) colorimetric method. *Total phosphorus*, in 2 to 5 cc. samples of Fractions 1 and 2, was determined as inorganic phosphorus after the following procedure. The sample chosen was boiled with exactly 0.5 cc. of concentrated sulfuric acid until white fumes appeared; it was then cooled and boiled with 1 cc. of distilled water and no more than 0.5 gm. of potassium persulfate until white fumes again appeared. Then it was cooled, diluted with 3 cc. of distilled water, and boiled for 10 minutes to decompose any excess persulfate. After cooling to room temperature, this solution was ready for analysis by the method of Leiboff. Standard phosphate solutions, with and without the prescribed treatment, usually matched; when they failed to do so, the attending results were rejected.

The foregoing methods are applicable to solutions containing uranyl ions in amounts equivalent to the samples mentioned. For the determination of phosphorus the method of Leiboff is preferred, because it is quick and convenient for a large number of analyses, while it gives the same results as the method of Tisdall, as shown in Table I.

In the ensuing experiments the hydrogen ion concentration was estimated colorimetrically by the use of Clark and Lub's series of indicators (11).

Results

The agents of disintegration of yeast nucleic acid were living bacteria; specifically they were members of the aerobic, spore-bearing group of bacilli. The three strains isolated were *Bacillus mesentericus vulgatus*, *Bacillus megatherium*, and *Bacillus subtilis*. Since the results for each strain were very much the same, those obtained with *Bacillus subtilis* were chosen as representative of group behavior.

Bacillus subtilis in its vegetative form grows rapidly on simple media, is a facultative anaerobe, and finds refuge in spores which resist boiling for hours. In spite of their rapid growth the amounts of total nitrogen and of total phosphorus contributed to the medium by the bacilli were very small compared to the amounts representing breakdown products of nucleic acid. This conclusion is demonstrated in Table II. In Tables II to VI each number represents the difference of results of analysis of a control mixture from those of a mixture containing bacteria.

Intensity of Nuclease Activity of Bacillus Subtilis—The most favorable pH for the nuclease activity of *Bacillus subtilis*, 6.6, is demonstrated in Table III, which presents results obtained from culture mixtures buffered at different pH values. Consideration of Table III reveals that the optimum pH for nuclease activity was 6.6, because corresponding values of total nitrogen and of total phosphorus in the uranyl chloride precipitates were lowest at that pH and because corresponding values in the uranyl chloride supernatant fluids were highest at that pH. The consistency of the P:N ratio and the value of the ratio, 0.59, which is also that of nucleic acid, indicate that none of the nitrogenous nor phosphoric products of nucleic acid had escaped as gases, such as ammonia.

TABLE I

Measurement of Free Inorganic Phosphorus in Uranyl Chloride Filtrates

Sample No.	Leiboff method	Tisdall method	Sample No.	Leiboff method	Tisdall method
	mg.	*mg.*		*mg.*	*mg.*
1	2.143	2.128	5	1.428	1.412
2	2.801	2.907	6	2.856	2.824
3	4.784	4.762	7	4.100	4.149
4	5.434	5.494	8	4.000	4.067

An environment of hydrogen, though it seems to accelerate phosphorus metabolism, is without influence on the nuclease activity of *Bacillus subtilis*. Substantiation for this statement is found in Table IV. One set of culture mixtures and one set of control mixtures, in tubes without stoppers, were exposed to hydrogen in a 2 liter glass jar from which oxygen had been extracted by a vacuum pump and by oxidation of an electrically heated copper wire attached to the lead cap sealing the jar and in which atmospheric pressure had been restored by intake of hydrogen. Duplicate sets of culture mixtures and of control mixtures were exposed to air at atmospheric pressure in a twin jar. Both jars were sealed and placed in an incubator at 37.5° for 48 hours.

Table IV shows, except for total and inorganic phosphorus at pH 6.0 and 6.6, that the nuclease activities of *Bacillus subtilis* were the same in an atmosphere of low oxygen tension as in an atmosphere of air. The discrepancies must have represented inorganic phosphorus, since the total nitrogen and organic phosphorus were the same for each environment; they were accounted for by examination of the uranyl chloride precipitates which showed that the total phosphorus of the anaerobic precipitate (Fraction 1) at pH 6.0 and 6.6 was greater than that of the aerobic precipitate of the same fraction by almost the exact amounts of the discrepancies, and further that no phosphorus could be

TABLE II

Contribution of Living Bacteria to Nitrogen and Phosphorus of Culture Mixtures of Bacillus subtilis and Yeast Nucleic Acid

Nucleic acid, 3.2 per cent; pH 6.6; time of incubation at 37.5°, 2 weeks.

Culture mixtures	Total N			Total P			Bacterial N*	Bacterial P*
	Fraction 2	Fraction 1	Fraction 4	Fraction 2	Fraction 1	Fraction 4		
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Bacterial	12.54	5.95	18.49	7.41	3.13	10.54	0.99	0.19
"	14.88	4.07	18.95	8.79	1.85	10.64	1.45	0.29
Control	1.05	16.35	17.40	0.62†	9.71	10.33		
"	1.01	16.59	17.60	0.62†	9.75	10.37		

Fraction 1, carbonate solution of the uranyl chloride precipitate; Fraction 2, the uranyl chloride supernatant fluid; Fraction 3, the supernatant fluid after treatment with neutral lead acetate; Fraction 4, the unfractionated culture mixture.

* Bacterial N equals 18.49 − 17.50 and 18.95 − 17.50; bacterial P equals 10.54 − 10.35 and 10.64 − 10.35.

† Inorganic phosphorus.

detected without preliminary treatment of the precipitate with strong acid. These results suggest that the discrepancies of inorganic phosphorus represent substance ingested by the bacilli.

TABLE III

Optimum Hydrogen Ion Concentration for Disintegration of Yeast Nucleic Acid by Cultures of Bacillus subtilis at 37.5° for 48 Hours

Nucleic acid, 3.2 per cent; pH as shown; time of incubation, 48 hours.

pH	Total N		Nuclease activity	Total P		Nuclease activity	P:N ratio	
	Fraction 1 (*a*)	Fraction 2 (*b*)	(*c*)	Fraction 1 (*d*)	Fraction 2 (*e*)	(*f*)	Fraction 1 (*d*/*a*)	Fraction 2 (*e*/*b*)
	mg.	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*		
6.0	12.9	14.9	53.6	7.18	8.72	54.9	0.56	0.59
6.6	5.8	22.9	80.0	2.48	13.52	84.5	0.43	0.59
7.2	7.1	20.8	71.0	3.54	12.46	78.0	0.50	0.60
7.8	12.7	14.7	53.6	7.42	8.51	53.4	0.58	0.58
8.2	17.9	9.4	34.4	10.51	5.43	34.1	0.59	0 58

$c = b/(b + a) \times 100; f = e/(d + e) \times 100.$

See note below Table II for explanation of fractions.

TABLE IV

Nuclease Activity of Bacillus subtilis under Aerobic and Anaerobic Conditions

Nucleic acid, 3.2 per cent; pH as shown; time of incubation, 48 hours.

Uranyl chloride supernatant fluid

pH	Total N		Total P		Inorganic P		Organic P	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
6.0	10.06	9.86	5.92	4.75	2.67	1.54	3.25	3.21
6.6	15.62	15.45	9.19	8.17	3.82	2.44	5.37	5.73
7.6	12.99	12.75	7.64	7.49	0.71	0.68	6.93	6.81
8.2	9.44	9.40	5.43	5.47	0.51	0.40	4.92	5.07

Bacillus subtilis continues to disintegrate yeast nucleic acid equally well, whether it be subcultured from solutions of nucleic acid or from agar slopes. From colonies on an agar slope bacilli were transferred to a 4 per cent solution of yeast nucleic acid and thereafter kept vigorous at 37.5° by forty-five daily subcultures from each medium. The last generations were transferred to agar slopes, incubated at 37.5° for 24 hours, and from them culture mixtures with yeast nucleic acid were made in the way described for pH 6.6. Each result in Table V was obtained from a separate culture mixture and so this table emphasizes the accuracy of the methods of fractionation as well as the reduplication of results.

Since yeast nucleic acid contains 16.3 per cent nitrogen and 9.63 per cent phosphorus, according to Levene and Bass ((8) pp. 274, 286), then the intensity of disintegration of nucleic acid by *Bacillus subtilis* may be stated in terms of mg. of nucleic acid broken down in 48 hours by multiplying the total nitrogen in the uranyl chloride supernatant fluid by 6.13 and by multiplying the total phosphorus therein by 10.38. When these calculations are made from values in Table III, it will be seen that about 130 mg. of nucleic acid were disintegrated in 48 hours and that the 130 mg. represented 80 per cent of the available nucleic acid.

TABLE V

Effect of Culture Media on Disintegration of Nucleic Acid by Bacillus subtilis

Time of incubation at 37°, *days*	Inorganic P in uranyl chloride supernatant fluid				
	0	3	6	9	Average at 9 days
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
Agent of nucleic acid disintegration					
45th subculture from agar slopes	0.100	2.388	4.088	4.580	
	0.100	2.392	3.840	4.680	
	0.100	2.452	3.920	4.700	4.653
45th subculture from 4% yeast nucleic acid	0.100	2.300	3.892	4.220	
	0.100	2.328	3.972	4.120	
	0.100	2.440	3.900	4.040	4.127

Extent and Quality of Disintegration of Yeast Nucleic Acid by Bacillus subtilis—Since of the nitrogenous derivatives of nucleic acid, neutral lead acetate precipitates only nucleotides, the ratio of total nitrogen in Fraction 3 to that in Fraction 2 will indicate the proportion of nucleotides broken down after separation from nucleic acid. The breakdown of nucleotides can occur in three ways: (1) PRN → P + RN, (2) PRN → P + R + N, (3) PRN → PR + N. All three ways are included in the foregoing calculation.

It will be seen that processes (1) and (2) necessitate the separation of phosphoric acid, and therefore the ratio of inorganic phosphorus in Fraction 2 to total phosphorus in the same fraction will indicate the proportion of nucleotides broken down in both these ways. The other process (3) can then be calculated as the difference of per cent hydrolysis estimated from values for phosphorus from that estimated from values for nitrogen. The results of these calculations are presented in Table VI. Comparison of columns (*f*) and (*g*) in Table VI shows that the nucleotides were disintegrated mainly to phosphoric acid ester of ribose and nitrogenous bases. This is particularly striking at alkaline pH values: at pH 8.2 the nucleotides were decomposed in this way only. It is quite possible that part of or all the inorganic phosphorus liberated at acid and neutral pH values may have been due to the hydrogen ion concentration independently of the bacteria, because phosphoric acid esters of ribose decompose

TABLE VI

Extent and Quality of Disintegration of Nucleotides by Bacillus subtilis

Nucleic acid, 3.2 per cent; pH as shown; time of incubation, 48 hours.

pH	Total N		Nucleotide hydrolysis	Total P	Inorganic P	Nucleotide hydrolysis	
	Fraction 2	Fraction 3		Fraction 2	Fraction 2		
	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)	(*g*)
	mg.	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*	*per cent*
6.0	14.9	11.5	77	8.72	2.67	31	46
6.6	22.9	18.3	80	13.52	3.54	26	54
7.2	20.8	16.6	80	12.46	3.39	27	53
7.8	14.7	7.4	50	8.51	0.06	1	49
8.2	9.4	2.8	30	5.43	0.00	0	30

$c = (b/a) \times 100; f = (e/d) \times 100; g = c - f.$

c represents nucleotide hydrolysis of all three types; *f*, nucleotide hydrolysis of both types yielding inorganic phosphorous; *g*, nucleotide hydrolysis of the type yielding phosphoric acid ester of ribose and free nitrogenous base.

See note below Table II for explanation of fractions.

quickly ((8) p. 167). Subject to that possibility and to two limitations of the analytical methods, the process of disintegration of yeast nucleic acid by *Bacillus subtilis* may be written

$$\text{Nucleic acid} \rightarrow 4(\text{PRN}) \rightarrow 4(\text{PR}) + 4(\text{N}) \rightarrow 4\text{P} + 4\text{R} + 4\text{N}$$

The limitations of the methods are the failure to discriminate between pyrimidine and purine compounds and the lack of sugar analyses.

SUMMARY

1. Methods are described for the study of the process of disintegration of yeast nucleic acid.

2. These methods were applied to cultures containing yeast nucleic acid and each of three members of the aerobic, spore-bearing group of bacilli.

3. *Bacillus subtilis*, *Bacillus mesentericus vulgatus*, and *Bacillus megatherium* were found to be agents for a rapid, intensive disintegration of yeast nucleic acid, particularly at pH 6.6.

4. The extent of substrate disintegration by these bacteria was not influenced by growth in an environment of low oxygen tension.

5. The bacilli retain their disintegrative power whether subcultured many times from agar or from solutions of yeast nucleic acid.

6. A tentative scheme for the process of disintegration of yeast nucleic acid by *Bacillus subtilis* was deduced from the results of analysis.

BIBLIOGRAPHY

1. Sachs, F., *Z. physiol. Chem.*, **46**, 337 (1905).
2. Deutsch, W., *Z. physiol. Chem.*, **171**, 264 (1927).
3. Levene, P. A., and Dillon, R. T., *J. Biol. Chem.*, **88**, 753 (1930); **96**, 461 (1932).
4. Schmidt, G., *Klin. Woch.*, **10**, 165 (1931).
5. Hahn, M., and Geret, L., *Z. Biol.*, **40**, 135 (1900).
6. Iwanoff, L., *Z. physiol. Chem.*, **39**, 31 (1903).
7. Schittenhelm, A., and Schroter, F., *Z. physiol. Chem.*, **41**, 284 (1904). Schittenhelm, A., *Z. physiol. Chem.*, **57**, 21 (1908). Sivèn, V. O., *Arch. ges. Physiol.*, **157**, 21 (1914).
8. Levene, P. A., and Bass, L., Nucleic acids, American Chemical Society monograph series, New York (1931).
9. Buell, M. V., and Perkins, M. E., *J. Biol. Chem.*, **76**, 95 (1928).

10. Michaelis, L., *J. Biol. Chem.*, **87**, 33 (1930).
11. Clark, W. M., The determination of hydrogen ions, Baltimore, 64, 82 (1920).
12. Van Slyke, D. D., *J. Biol. Chem.*, **71**, 235 (1926–27).
13. Tisdall, F. F., *J. Biol. Chem.*, **50**, 329 (1922).
14. Leiboff, S. L., *J. Lab. and Clin. Med.*, **16**, 495 (1931).

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STUDIES ON THE ETIOLOGY OF SPONTANEOUS CONJUNCTIVAL FOLLICULOSIS OF RABBITS

II. BACTERIOLOGICAL INVESTIGATIONS

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PLATES 16 AND 17

(Received for publication, June 4, 1934)

Spontaneous conjunctival folliculosis, a disease widespread among rabbits, has been described in a preceding article (1) as occurring in two clinical forms, Type I and Type II. The former is characterized by a dormant chronicity with discrete lesions, whereas the second type, a more actively progressive disease, presents an inflammatory follicular reaction involving all the palpebral conjunctival surfaces. It was found that the transition from one type to the other, an infrequent occurrence in nature, could more often be induced artificially by experimental procedures. From our studies a conclusion was drawn that the causal factor of the affection is not an ultramicroscopic virus but an infectious agent behaving after the manner of bacteria of low grade pathogenicity.

In this second paper the results of our bacteriological investigations will be presented.

Methods

The methods employed in cultivation tests were similar, with the exceptions to be noted, to those in bacteriological studies on spontaneous monkey folliculosis (2). The exceptions consisted in the use of: (*a*) defibrinated rabbit blood instead of horse blood in the preparation of the blood agar medium, a substitution made merely for the sake of convenience; (*b*) dextrose instead of five carbohydrates, of which a 5 per cent solution was filtered through a Berkefeld V candle, and 5 cc. of the sterile filtrate were added to 95 cc. of the blood agar; (*c*) agar containing 2 per cent Witte's peptone, adjusted to pH 7.4, cooled to 45°C., and into this sterile, defibrinated, rabbit blood was introduced to make 8 per cent by volume.

Although plain dextrose agar readily supported the growth of the several species of bacteria that were cultivated, the employment of blood agar was found to be preferable, especially for initial cultures, in that it afforded a more profuse growth of fragile organisms and facilitated their recovery. For each test six plates of blood agar and four tubes of leptospira medium, prepared as already described (2), were seeded with saline solution suspensions of excised conjunctival tissue.[1] The excised tissue, rather than expressed follicular, or grattage material, was found to be more advantageous for bacterial cultivation.

Cultures of Affected Tissue and Control Materials

The bacterial flora of the conjunctival tissue obtained from normal rabbits was not unlike that from monkeys (2). Staphylococci and diphtheroids predominated, whereas the microorganisms less frequently encountered were the Gram-negative, motile or non-motile, chromogenic or non-chromogenic bacilli of indeterminate species; organisms of the *subtilis* group; yeasts and moulds. The relative incidence of these bacteria in normal tissue was low, and in affected conjunctivae much higher. *Bacterium granulosis* and *Bacterium simiae*, associated with human or experimental trachoma (3), and with simian folliculosis (2), respectively, were not found. Nor could we isolate *Bacterium monocytogenes* (3, 4), the incitant of fatal epizootics among rabbit breeding stocks.

Thirty-eight strains of the indeterminate group of organisms were collected, and of these, sixteen different cultures were injected into 46 rabbits in the manner already described (1). It should be noted that characteristic folliculosis was not induced thereby, although in some instances transitory, diffuse abscess formation resulted and in others localized pustules at the points of scarification. In all cases healing was prompt and normal conditions were restored within a week or two after inoculation.

The different species of ordinary or indeterminate bacteria, as mentioned, were not the only ones which were isolated from the rabbit folliculosis tissue. In addition to these, we have cultivated from affected conjunctivae a new species of microorganism which, after inoculation into rabbits, induced a conjunctival follicular reaction indistinguishable from the spontaneous disease as it occurs in nature. This special bacterium has been isolated from seventeen of 24 rabbits showing fully developed, diffuse (Type II) folliculosis. There was, however, considerable difficulty in cultivating it from animals having the Type I disease, which is less readily transmissible to normal rabbits (1), and from those showing a mild degree of the diffuse form of the affection. For example, only five positive cultures were obtained from eighteen such animals.

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[1] All operations on animals were performed with the aid of ether anesthesia.

Description of the Microorganism

In the following brief form will be presented the chief morphological and biological characteristics of the special microorganism isolated and cultivated from conjunctival folliculosis of rabbits.

Morphology.—The common form of the microorganism is a slender, thin bacillus, with pointed ends, two or three times longer than its width, and ordinarily smaller in size than *Bacterium granulosis* or *simiae*, or 0.2 to 0.3μ wide and 0.5 to 1μ long. The appearance of the minute, delicate, tenuous bacilli in stained film preparations is not unlike that of *H. influenzae* (Fig. 1). It is pleomorphic; one often sees shorter coccoid or lanceolate structures frequently occurring in pairs, placed end to end. As a rule, smaller organisms preponderate in growths in leptospira medium and larger ones on agar.

Staining Reactions.—The organisms are Gram-negative and non-acid-fast. With stains for capsules, especially those of Muir or Casares-Gil, a faint, delicate envelope can be observed outside of a thin halo surrounding the bacilli or the diplo-lanceolate forms. The capsule is of much finer texture than the membranes surrounding the *granulosis* or *simiae* species, and it is not demonstrable by ordinary tinctorial methods. The Casares-Gil stain also reveals peritrichal flagella which apparently proceed from the capsular material (5). One flagellum is attached to each pole and one, two, or three, usually two, to each side. The thready structures are long, tenuous, and wavy and in stained film preparations often appear matted or thrown to one side (Fig. 2).

Motility.—The multiple flagella contribute to the peculiar and highly active motility of the organism. The bacilli move rapidly in a circular or linear direction with a motion that may be either waddling or tumbling. There are some, however, that revolve about their central points as axes, like a pinwheel, without forward progression. The motility is not lost with the age of a culture, nor is it influenced by the medium employed.

Spores and metachromatic granules are absent.

Agar Plates.—The colonies first appear in 24 to 48 hours as minute, smooth, spherical growths about 1 mm. in diameter, enlarging in 24 to 48 hours to a size of 2 or 3 mm. They are translucent, somewhat convex, moist, and mucoid. The edge is entire; the substance is slightly greyish and homogeneous. There is a tendency of one colony to coalesce with its neighboring one (Fig. 3). When magnified at 10 diameters as with a colony microscope, it exhibits a peaked center and a sharply defined, but slightly raised edge (Figs. 4 and 5). The growth on blood agar is more profuse and the colonies are somewhat less translucent and more greyish. As occurs with *granulosis* and *simiae* microorganisms, cultures of affected conjunctival tissue yield only an occasional colony of the special bacterium among many miscellaneous growths.[2] The colonies are sometimes adherent to, or incorporated within an alien one. In the latter instances, mixed growths can be purified by repeated subplants on fresh media.

Agar slants exhibit within 48 hours a slightly greyish, translucent, coalescent growth, which is glistening, mucoid, homogeneous, and non-spreading. The water of syneresis appears uniformly cloudy or milky, depending on the amount of growth.

Leptospira Medium.—24 hours after inoculation, a faint nebulosity occurs just at the surface. Following this there is an ingrowing, sac-like mass, with its base, 5 mm. across, lying at the center of the under surface and extending for 5 mm. into the medium. The area spreads out laterally so that within a day or two there is a uniformly opaque, whitish layer up to 1 cm. high. A slowly progressive extension of the clouding takes place until the bottom of the tube is reached, usually within 7 days.

Gelatin.—No liquefaction occurs. In stab cultures one observes a tenuous, arborescent, non-spreading line of growth. Colonies on gelatin agar appear greyish, mucoid, and confluent but they are less numerous than on blood agar.

Broth.—In this medium the growth is uniformly turbid without pellicle formation.

Litmus Milk.—Unchanged.

Potato.—Faint, buff colored (changing to brown after 5 days), non-spreading, sparse surface culture.

Indol.—Not produced.

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[2] A probable explanation for the sparse colony distribution of the three species of organisms in initial cultures of affected tissues is that the medium may not be completely satisfactory for primary isolations, or the organisms may be suppressed by the growths of the rapidly developing, concomitant, miscellaneous bacteria present. Indeed, it has been shown by Weiss that the growth of staphylococci *per se* chiefly by lowering the pH of the medium definitely inhibits the development of *Bacterium granulosis*—the degree of inhibition being measurable on a quantitative basis. Diphtheroids also suppress *granulosis* growth but not as markedly (6). As already indicated, staphylococci and diphtheroids are predominating organisms in cultures of affected conjunctival tissues. We have also observed the inhibition, under these conditions, of growths of *granulosis*, *simiae*, and the special rabbit bacteria. (See also Reference 7; and for a recent discussion of the principles underlying bacterial antagonism, Neufeld, F., and Kuhn H., *Z. Hyg. u. Infektionskrankh.*, 1934, **116**, 95.)

Nitrates.—Not reduced.

Carbohydrate Reactions.—This organism is non-fermenting in type. No acid or gas is produced on dextrose, levulose, mannose, mannitol, saccharose, raffinose, inulin, galactose, maltose, salicin, xylose, dextrin, arabinose, amygdalin, lactose, dulcitol, rhamnose, trehalose, sorbitol, or inositol.

Other Properties.—The bacterium is aerobic and facultatively anaerobic. It produces no characteristic odor in cultures and is bile-resistant. The optimum temperature for development is 28–30°C. The thermal death point is at 56°C. for 15 to 30 minutes.

Filtrability.—Cultures suspended in saline solution failed, in ten tests, to pass through single disc Seitz and Berkefeld V filters. Similar material suspended, however, in hormone broth was filtrable through three of the four Berkefeld V filters used but not through Seitz discs. Precisely similar results were obtained when cultures of *Serratia marcescens* (*Bacillus prodigiosus*) were employed. The filtrability of many species of bacteria is possible in a hormone broth suspension and the results of tests with the special bacterium do not necessarily indicate that it has a filtrable stage or that it is a "filtrable" organism. However that may be, we made use of this as a practical finding in selective filtration through pervious Berkefeld V candles of mixtures of the bacterium with larger, non-filtrable organisms, such as staphylococci, so as to facilitate its recovery in a pure state from initial mixed cultures. The methods followed have already been described in similar experiments with *Bacterium granulosis* (7).

Serological Reactions.—Repeated intravenous injections into rabbits of living cultures of the special bacterium yielded antisera with an agglutinin titer of 1:1000 to 1:20,000 for all the available cultures, 22 in number, of the microorganism. The specificity of the agglutination was shown by the negative reactions in 1:10 dilution of the sera in tests with ordinary bacteria and with 37 strains of the miscellaneous, indeterminate bacteria recovered from rabbit conjunctivae. At this point mention should be made of the fact that *Bacterium granulosis* and *simiae* exhibit no cross-agglutination with the special bacterium. Hence the rabbit organism is serologically specific.

No agglutinins against this bacterium were found in 1:2 to 1:15 dilutions of the sera obtained from 29 normal rabbits. On the other hand, thirteen of fourteen animals infected by subconjunctival inoculation of the organism, as will be immediately described, revealed specific clumping in 1:20 to 1:160 dilutions of serum in tests made during the 3rd to 6th week after inoculation. Nevertheless, the organism was not agglutinated by the serum of rabbits having spontaneous folliculosis or the experimental disease evoked by injection of affected tissue. By comparison, no agglutinins were found in trachoma serum against *Bacterium granulosis* (8), nor in monkey folliculosis blood against *Bacterium simiae* (2). Moreover, Weiss (9), in a summary of his own and prior reports on localized infections of the conjunctiva, a class that includes rabbit folliculosis, has concluded that such affections do not induce appreciable antibody formation.

Thus in the general properties as given, the bacterium isolated and cultivated from cases of rabbit folliculosis appears to be distinct from other described microorganisms. It has, however, the same generic characteristics as *Bacterium granulosis* and *Bacterium simiae*. The diagnosis, or characters, of this genus may be stated as follows:

Small, slender Gram-negative rods present in the conjunctiva of man and animals affected by a follicular type of disease; mucoid type of growth which in early subplants takes place with some difficulty in ordinary media; motile, flagellated, and encapsulated; aerobic and facultatively anaerobic, produce no endospores, optimum temperature for growth 28–30°C.

On the other hand, the rabbit microorganism differs in specific characters from those of either the human or simian bacterium, in the degree of motility; in the arrangement of flagella; in the manner of growth on the same medium; in the reactions with carbohydrates, and especially in serological specificity, so that the designation of the rabbit bacillus as a distinct species is indicated.

Pathogenicity Experiments

Sixteen cultures of the special bacterium were available for pathogenicity tests.

Of the sixteen cultures, fourteen individually and two pooled were injected into anesthetized animals. The 48 hour growth on agar slants of a second, or more remote generation, was suspended in 1 cc. of 0.9 per cent saline solution. 0.2 cc. of the suspension was introduced subconjunctivally into young rabbits having smooth lids. These animals were selected and quarantined in the manner already described (1). Only the left lower membrane received the culture and two rabbits were employed for each strain; hence 30 rabbits were employed for the sixteen cultures.

Only one strain was inactive; the remaining fifteen induced conjunctival follicular reactions in all but two of 28 inoculated animals. The resultant experimental disease could not be differentiated either clinically or histopathologically from the natural affection or from the infection evoked by inoculation of folliculosis tissue itself (1). In all instances the disease developed eventually into diffuse, Type II folliculosis (Figs. 6 to 8) and from six of such animals the identical organism was recovered. These latter strains were again shown to be specifically active in normal rabbits.

Instillation of cultures of the special bacterium into the conjunctival sac of normal rabbits, in accordance with the method used previously (1), also brought about the same type of specific infection in two of three animals. These two exhibited characteristic folliculosis, 11 and 18 days respectively after the last instillation. Furthermore, the same microorganism was recovered from the conjunctivae of both, in the 3rd and 7th week of their illness, when folliculosis was fully established.

Microorganisms in Tissues.—The stained sections of lesions induced by the special bacterium and film preparations of expressed follicular material were studied. In the latter instance, the Giemsa- or Gram-stained grattage material obtained from 22 rabbits showed in ten of them miscellaneous bacteria varying in number and consisting chiefly of staphylococci and diphtheroids. In addition, in all the specimens were observed discrete or clumped, free or intracellular, Gram-negative bacteria which closely resembled the special microorganism in morphology (Fig. 9). An examination of sections of over twenty different follicular conjunctivae, stained by the Giemsa, Gram, or eosin-methylene blue methods, yielded similar results (Figs. 10 and 11). The microorganism, however, was found in such material to be smaller and often intracellular. It is of interest that the bacteria were seen in section or film preparations that were derived from rabbits having either the natural disease, or experimental folliculosis induced by inoculation of the special bacterium and of conjunctival tissue. Finally, cellular inclusion bodies that are characteristically associated with the action of many ultramicroscopic viruses were not observed.

SUMMARY AND CONCLUSIONS

Before summarizing the experimental results presented herewith, a brief review is indicated of our study on follicular conjunctivitides existing in the different species of animals thus far examined.

Spontaneous follicular conjunctivitis of monkeys and chimpanzees, which resembles the early, or follicular, stages of human trachoma, has been shown to be an infectious disease, and to arise from the action of a bacterium and not an ultramicroscopic virus (2, 10). A new species of organism, designated tentatively as *Bacterium simiae, n.sp.*, and capable of reproducing granular lids in normal monkeys and apes, has been recovered from cultures of simian folliculosis tissues. This organism, although of a different species, is of the same genus as *Bacterium granulosis* which is associated with human trachoma (3).

The studies were extended beyond folliculosis in monkeys and apes to a similar disease widespread among stock rabbits. Its clinical appearance and microscopic changes resemble the spontaneous malady in simians with some minor differences (1). Like the latter, rabbit folliculosis was also shown to be infectious and contagious.

In the present paper a bacteriological investigation of the rabbit affection is described. In the experiments undertaken we followed the method used by Noguchi in his studies on trachoma, that is, the different organisms recovered from normal and affected conjunctivae were inoculated into the conjunctivae of selected, quarantined rabbits.

Among the bacteria thus isolated from folliculosis tissue a new species was disclosed that brought about conjunctival reactions apparently indistinguishable either from the disease seen in nature or from the experimental infection induced by inoculation of folliculosis tissue. The organism was specifically active in rabbits whether injected subconjunctivally or instilled into the conjunctival sac. Furthermore, the special bacterium has thus far been cultivated only from folliculosis cases and not from other forms of conjunctivitis nor from normal tissues. Finally, specifically pathogenic strains of the identical microorganism have been recovered from rabbits experimentally infected with the bacterium.

A distinctive feature of this bacterium is that it is of the same genus as the microorganisms heretofore designated as *Bacterium granulosis* and *Bacterium simiae.* It is clearly evident then that the three bacteria cultivated to this time form a new genus, for which we propose the name *Noguchia.* The diagnosis of the genus has already been described in this paper, but as was also shown in a foregoing section, certain specific characters differentiate the three. Accordingly, we suggest a change in the names of the *granulosis* and *simiae* species to the proposed binomials, *Noguchia granulosis, n.sp.*, and *Noguchia simiae, n.sp.* The specific organism, having an evident causal relationship to rabbit folliculosis, can therefore be placed in this classification, with the name of *Noguchia cuniculi, n.sp.*[3]

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[3] The classification of genus and species follows the recommendations of the Society of American Bacteriologists quoted by Bergey, D. H., in Manual of

In conclusion it would appear that in three types, at least, of follicular reactions in the conjunctiva—in man (the primary lesion of trachoma consisting essentially of follicles), in the simian, and in the rabbit—there is an intimate association of microorganisms having the same generic, but different specific properties.

BIBLIOGRAPHY

1. Olitsky, P.K., Syverton, J. T., and Tyler, J. R., *J. Exp. Med.*, 1934, **60**, 107.
2. Olitsky, P. K., Syverton, J. T., and Tyler, J. R., *J. Exp. Med.*, 1933, **57**, 871.
3. Noguchi, H., *J. Exp. Med.*, 1928, **48**, suppl. 2.
4. Murray, E. G. D., Webb, R. A., and Swann, M. B. R., *J. Path and Bact.*, 1926, **29**, 407.
5. *Cf.* Churchman, J. W., and Emelianoff, N. V., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 996.
6. Weiss, C., personal communication.
7. Knutti, R. E., Olitsky, P. K., and Tyler, J. R., *Science*, 1931, **73**, 709.
8. Olitsky, P. K., *Tr. Am. Acad. Ophth. and Oto-Laryngol.*, 35th Ann. Meeting, October 31, 1930, 225.
9. Weiss, C., *J. Immunol.*, 1933, **25**, 259.
10. Olitsky, P. K., and Tyler, J. R., *J. Exp. Med.*, 1933, **57**, 229.

EXPLANATION OF PLATES

PLATE 16

FIG. 1. Gram's stain. Agar slant culture. ×1000.

FIG. 2. Casares-Gil stain, showing flagella; the staining fluid has thrown the structures to one side of the bacterium. ×1000.

FIG. 3. 48 hour growth on blood agar plate. Natural size.

FIG. 4. Same colonies photographed by reflected light. ×10.

FIG. 5. Same colonies photographed by transmitted light. ×10.

FIG. 6. Upper and lower conjunctivae of rabbit inoculated with a culture of *Noguchia cuniculi*. The characteristic folliculosis thus brought about should be compared with the condition seen in nature, as described and illustrated in the preceding paper (1). Natural size.

PLATE 17

FIG. 7. Eosin and methylene blue stain. Conjunctiva obtained from a rabbit inoculated subconjunctivally with the microorganism. The induced lesions are

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determinative bacteriology, and by Buchanan, R. E., in General systematic bacteriology, both published by Williams & Wilkins Co., Baltimore, 1925; also the Code of the Nomenclature Committee of the International Society of Microbiology (1930) quoted in Bergey's Manual, as stated, 1934, 4th edition, p. 23.

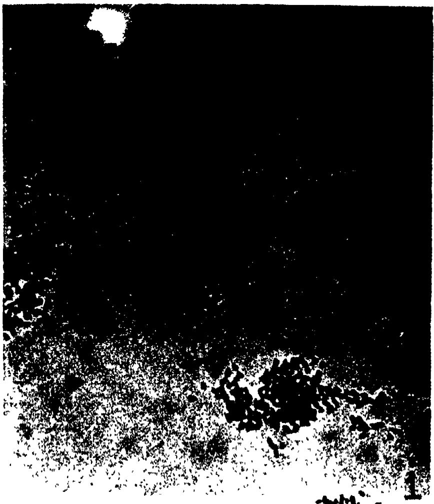
identical with those found in the natural disease or the experimental infection caused by injection of affected tissue, as illustrated in Paper I (1). ×122.

FIG. 8. Eosin and methylene blue stain. Same as Fig. 7. Represents only a part, about a third, of the entire follicle. ×300.

FIG. 9. Giemsa stain. Film preparation of conjunctival grattage material obtained from a rabbit having spontaneous folliculosis. Among miscellaneous microorganisms are seen bacteria with the morphology of *Noguchia cuniculi.* ×1000.

FIG. 10. Eosin and methylene blue stain. Tissue section of spontaneous conjunctival folliculosis in a stock rabbit showing a clump of similar bacteria within a cell. ×1000.

FIG. 11. Same. ×1500.



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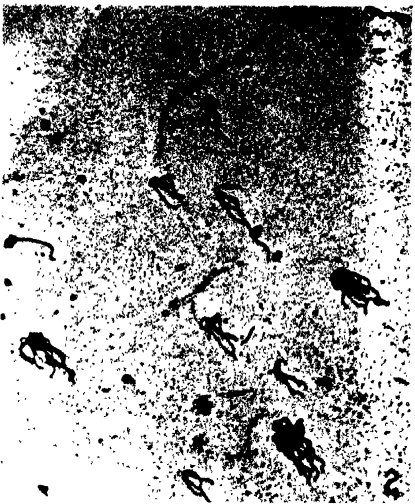

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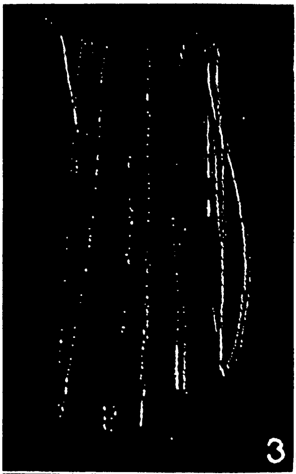

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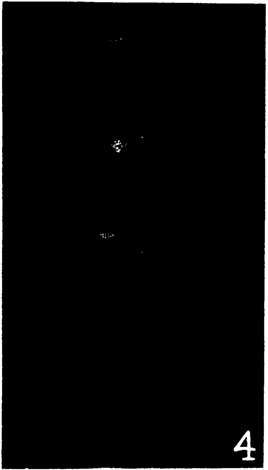

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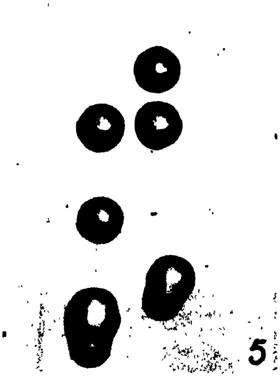

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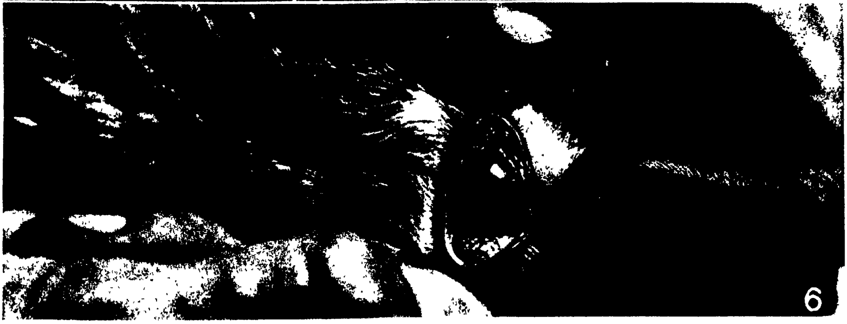

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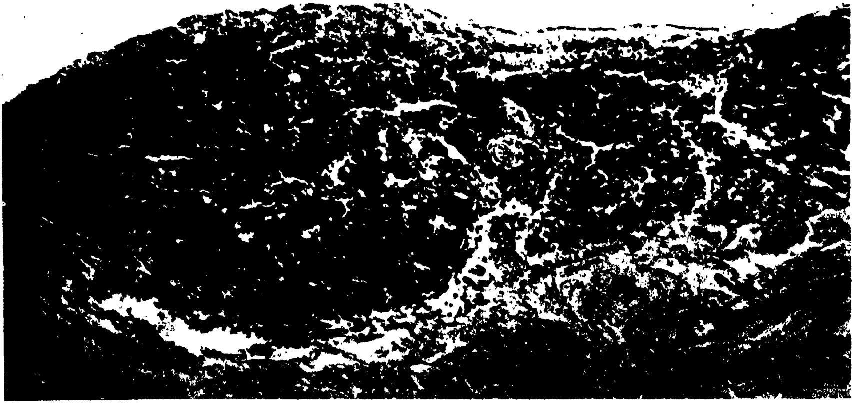
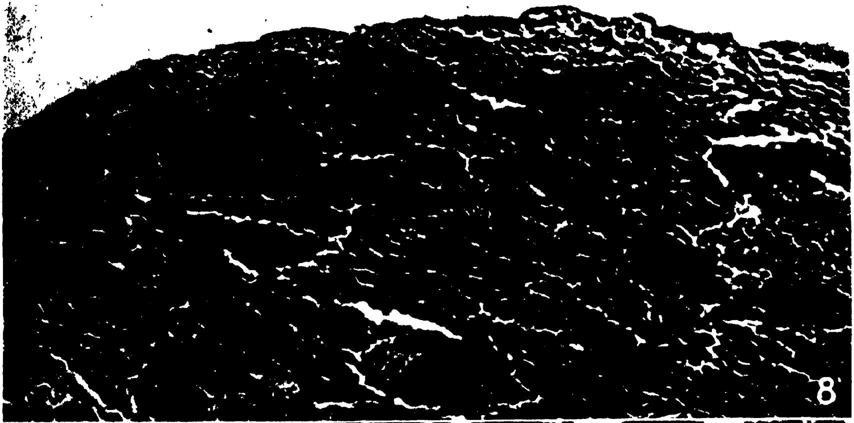
Photographed by Louis Schmidt

(Olitsky *et al.*: Conjunctival folliculosis of rabbits. II)





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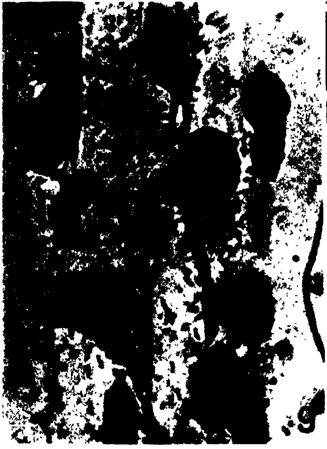

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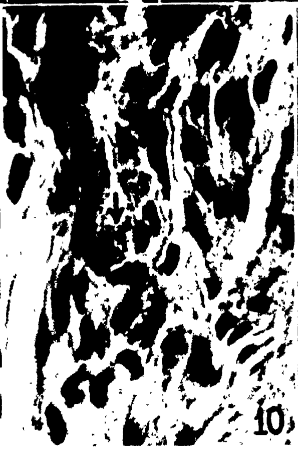

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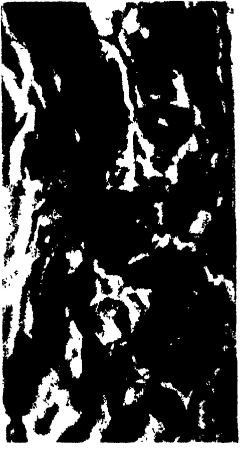

Photographed by Louis Schmidt

(Olitsky *et al.*: Conjunctival folliculosis of rabbits. II)

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BACTERIOLOGICAL STUDIES ON AN EPIZOOTIC OF INTESTINAL DISEASE IN SUCKLING AND NEWLY WEANED MICE

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(Received for publication, June 15, 1934)

In August, 1933, a spontaneous epizootic, with clinical signs of an acute intestinal affection, first appeared among the mice in the Rockefeller Institute breeding stock.[1] Previous epizootics caused by Salmonella infection have occurred at the Institute and have been described by Flexner (1) and his associates, Lynch (2), Amoss (3, 4), Webster (5, 6), and Amoss and Haselbauer (7). The present affection, however, was distinctive in its sharp limitation to suckling and newly weaned mice, as well as in difference in the stock affected, symptomatology, pathological changes, and in basic etiological origin. Epizootics similarly confined to the corresponding age group of animals and comparable in some other respects to the outbreak studied by us have previously been described (8–14).

For example, natural infection of hogs with *Salmonella suipestifer* is restricted to the very young, usually suckling or newly weaned shoats. "All authorities appear to agree that such infection does not occur naturally except in very young subjects" (8). When older animals present similar symptoms the disease is usually the virus affection known as hog cholera or swine fever. The fact that there is an independent, but similar, affection due to Salmonella infection would support the early investigations of Salmon and Theobald Smith (1885), and Welch (9) in their studies on hog cholera bacillus (8).

In this paper we shall record the clinical and pathological appearance of the disease in mice, its epizoology, and the results of bacteriological studies.

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[1] The mice raised in The Rockefeller Institute for experimental purposes consist of two strains: an albino strain inbred for 22 years, known as the Rockefeller Institute strain, and a strain of Swiss mice. The former is bred and used in greater numbers than the latter.

Clinical Signs

The malady was confined to suckling and newly weaned mice 7 to 24 days old. There was profuse diarrhea attended by apparent tenesmus and marked inanition. Loss of weight and tone was extremely rapid; complete prostration occurred within 24 to 72 hours after the initial signs and death took place in about one-third of the animals affected. Obstipation and cessation of suckling sometimes supervened, leading to a dehydration that was almost uniformly fatal. The obstipation was noted in about one-third of the mice. In the small number that recovered after showing this complication, necrosis of the tissues of the perianal region with sloughing frequently occurred. In the absence of obstipation, recovery was usually rapid and complete; after several days the recovered mice were indistinguishable from unaffected litter mates.

Pathology

The gross lesions are limited largely to the intestinal tract and vary from slight hyperemia to frank necrosis of the lower ileum and colon. The spleen is dark red and contracted; the hyperemic tumefaction so frequently seen in mouse typhoid is not present. On the other hand, in recovered mice the spleen may show gross cicatrization.

Microscopically, the most characteristic changes are found in the lower ileum and colon. The mucosa is infiltrated with polymorphonuclear and red cells; it may exhibit generalized ulceration. The mucosa is often found as a slough in the lumen of the gut. The lymphoid tissue is increased, with hypertrophy of Peyer's patches particularly marked. The involvement of the gut is generalized and is not restricted to circumscribed ulcerations.

The spleen is hemorrhagic and contains many clasmatocytes. The pulp cells show varying stages of necrosis. A diffuse, parenchymatous degeneration of the liver occurs in varying degree, being somewhat more pronounced in the periportal areas. In marked cases there is a fatty degeneration of the liver parenchyma associated with localized hemorrhages. Only exceptionally does one observe focal necrosis of groups of liver cells. The kidneys reveal congestion and localized hemorrhages with early glomerular changes and granular degeneration of the tubular epithelial cells. An occasional, minute, focal hemorrhage is usually found in the brain and with this exception the organ appears unchanged.

In general the lesions consist of ulcerative enteritis; hemorrhagic and necrotic splenitis; fatty degeneration of the liver, and acute parenchymatous nephritis.

Epizoology

Epizoological observations were largely limited to the time during which the experimental work was in progress. The caretakers of the breeding room, however, had kept a record of the number of infected mice discarded prior to the time our investigations began, thus enabling us to estimate closely the number of animals affected.

The disease, as previously mentioned, was confined to infant mice, 7 to 24 days old. It quickly spread in sporadic outbursts throughout the entire colony, with a somewhat higher morbidity among the Swiss than the Institute strain. From 15 to 100 per cent of an individual litter would become sick; the average morbidity approximated 75 per cent, and of these 60 per cent died. In any cage litter mates that did not show signs of the illness within the first 48 hours after onset would invariably maintain their resistance completely. Their sleek, healthy, general appearance and activity were in striking contrast to those of their sick or recovering siblings.

The epizoological curve in this disease was directly comparable to that described by Lynch (2). The peak was in September, at which time the case incidence reached 35 per cent of an infant population of 4,000. Following this, the incidence gradually receded to a low point in December and January, at which time 11 per cent of 2,900 were ill. A secondary rise in February to 35 per cent of 3,800 infant mice was then observed with a subsequent abatement that is again approaching the previous low level at the time of writing. The occurrence of the two points of highest case incidence, namely, September and February, corresponds to those found by Pritchett (15) in her studies on the seasonal variation in the susceptibility of five strains of mice to infection *per os* with *B. pestis caviae*, a causal agent of mouse typhoid.

Preliminary Investigations

Investigations were first undertaken to determine possible etiological factors.

Diet.—The routine diet used in the mouse room throughout the past 15 years consists of a teaspoonful of stale white bread soaked in fresh, pasteurized, Grade B milk for each mouse daily, and is supplemented by a mixture of equal parts of oatmeal and buckwheat twice a week and by dog biscuit once a week. Substitutes for the various components of this diet, as well as substitutions of entirely different diets under well controlled conditions, were without effect in reducing the incidence of the disease, except in one particular, the details of which follow.

The extent of the morbidity and mortality was found to be dependent to a greater or less degree on the amount of water imbibed by pregnant females.[2] This is illustrated by the following protocol.

	No. of pregnant mice	No. of mothers showing infected litters	No. of young affected	No. of affected young that died
Given water with diet......	20	5	14	1
Without water.............	20	6	20	11

On the other hand, the epizootic continued when water was plentifully supplied to nursing mothers, so that the absence of this factor can be considered only as contributing to the gravity of the disease.

Contact Infection.—The infectious nature of the disease was determined by contact experiments in which sick mice were placed among normal, quarantined mice.[3] In four such experiments, in which 24 normal mice were used, 30 per cent of them developed the characteristic enteritis. Thus the resultant morbidity in the individual litters approximated that found in the epizootic as it occurred in the breeding room.

Virus.—A virus as the cause was eliminated as follows:

Repeated filtration tests were made: the filtrates of viscera, brain, and intestinal contents, as well as emulsions of spleen, liver, and brain subsequently found to be bacteriologically sterile were inoculated by intraperitoneal, intracerebral, subcutaneous, and intramuscular routes without apparent effect. Furthermore, there was an absence, in especially prepared and stained sections of affected tissue, of specific cytotropism or inclusion bodies, such as are commonly encountered in many ultramicroscopic diseases.

Protozoa.—Repeated stool examinations and a study of pathological sections of the intestines and liver excluded a protozoal infection.

Bacteriological studies now became more promising and it is to this aspect of the problem that the remainder of this paper will be devoted.

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[2] We are indebted to Dr. L. Whitman of the Rockefeller Foundation for his helpful suggestions as concerns this experiment.

[3] Impregnated Swiss females were obtained from an outside source and kept in rigid isolation in a room apart from those used for experimentation. They and their offspring were employed in this and in the other experiments to be described.

Bacteriological Methods

Heart's blood, emulsions of spleen, liver, kidney, and brain, and contents of the different divisions of the large and small gut were used as the inocula for bacteriological cultures. The media employed consisted of blood agar and Endo plates, a modification (16) of the leptospira media of Noguchi, and hormone broth.

Results of Cultivation Tests

Twenty-six mice showing typical signs of infection were used for cultivation purposes. From twenty, or 77 per cent, of these an organism was recovered the major characteristics of which definitely place it in the genus Salmonella. This organism was isolated from the intestine in every instance, and also from the heart's blood on three occasions, and the liver and spleen, respectively, on four occasions. No other species of organism was recovered from the liver, spleen, or heart's blood.

The number of colonies on Endo plates was highly variable, ranging from one to two on several plates inoculated with material from the same mouse to almost pure cultures. It was observed that the colonies were more numerous and predominated over those of other bacteria at the height of the epizootic.

Similar cultivation tests carried out on mothers of infected litters, including the mammary glands and excreta, yielded entirely negative results.

Description of the Microorganism Recovered

Morphology and Staining Reactions.—It is a non-spore-forming, small bacillus 1 to 3 μ in length and 0.4 μ to 0.7 μ in diameter. It is readily stained, Gram-negative, and although it usually appears discrete it may be seen in very short chains.

Growth Characteristics.—A variety of media readily supports its growth. Plain broth and agar slants, pH 7.4, show an abundant growth in 18 hours. Gelatin is not liquefied. Indol is formed. There is slight acidification but no coagulation of litmus milk. Nitrates are reduced to nitrites.

In carbohydrate broth no acid or gas is formed in lactose, saccharose, raffinose, inulin, salicin, dextrin, amygdalin, or inositol. Acid and gas are formed in glucose, levulose, mannose, galactose, arabinose, rhamnose, maltose, xylose, trehalose, and sorbitol. The reactions in mannitol and dulcitol were slightly irregular with only an exceptional culture not forming acid and gas. Like other Salmonella, the special organism produces no clouding of lactose broth in the closed branch of the fermentation tube devised by Theobald Smith. The hydrogen sulfide and the Voges-Proskauer reactions are negative.

Serological Reactions.—Rabbit antisera were obtained that had an agglutination titer up to 1:12,800 for thirteen strains of the bacterium. The remaining seven strains recovered were all a variant that did not give the same agglutination titer but did conform in respect to sugar reactions, production of indol, and effect on litmus milk. Such serological variants are known to occur in natural or induced Salmonella infections in mice (4, 17, 18, 19). Rabbit antisera prepared from the variant organism showed agglutination in a dilution of 1:80 with the type bacterium.

Specific agglutinins were demonstrated in serum from each of 23, or 34 per cent, of 67 recovered mice with two having an agglutination titer of 1:640, three 1:160, five 1:80, four 1:40, eight 1:20, and one 1:10. Agglutinins for the variant were present in the same percentage of recovered mice. Agglutinins were also demonstrated in the serum of mothers having litters 100 per cent affected, in that two out of twelve gave agglutination in titers of 1:40 and 1:320.

It is noteworthy that agglutinins were not present in serum from normal quarantined mice; none of 50 showed agglutination.[4] As a further control, a duplicate set-up was made using the mouse typhoid organism, M.T. II (20) or *Salmonella aertrycke*, with which entirely negative results were obtained with individual sera from 27 recovered mice, and from seven mothers having infected litters.

A comparison with results obtained by other investigators in the related mouse typhoid epizootics is of interest. For example, Amoss (4) found that 22 of 56 survivors showed complete agglutination with *Salmonella aertrycke* cultures in 1:40 to 1:160 dilutions of serum. In one of Webster's experiments (21) in which 100 mice were artificially infected with *B. pestis caviae* seven of 26 survivors revealed agglutination with the homologous organism. Topley and Ayrton (22) and Topley, Ayrton, and Lewis (23) reported agglutinins in six of 68 in one series of induced infections and in seven of 55 in another. Hence the incidence of agglutinins was less than in the case of Amoss and greater than in that of the other investigators mentioned.

Experiments on Pathogenicity

Subcutaneous and Intraperitoneal Inoculation.[5]—In determining the pathogenicity of the recovered organism a preliminary experiment demonstrated that, with a saline suspension of an 18 hour agar slant growth, 100 million organisms were uniformly fatal when injected intraperitoneally or subcutaneously into infant mice, all of 24 dying within 48 hours. In titration of a similar preparation, 10,000 bacteria were found to kill, whereas 1,000 organisms produced a non-fatal enteritis. The induced experimental disease showed pathological changes indistinguishable from the natural affection. The organism was again recovered from the heart's blood, liver, and spleen of artificially infected mice in every instance.

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[4] We are indebted to Dr. L. T. Webster for several samples of normal mouse serum.

[5] Ether anesthesia was used for all operative procedures.

Intraperitoneal administration of the same organism to ten recovered mice in the massive dose of 150 million organisms resulted in the death of only four, or 40 per cent. The disease was more protracted with death ensuing after as long as 15 days. Apparently a relative immunity to the microorganism had been acquired as result of a previous attack of the disease.

Ingestion of Organisms.—Characteristic experimental enteritis could be reproduced in infant mice by intraoral administration of cultures of the bacterium. Thus four of twelve animals receiving orally the cultures once on each of 3 consecutive days became affected.

Effects on Adult Mice.—Only relatively massive doses, that is, from 100 to 300 million organisms, induced fatal enteritis on intraperitoneal injection into adult mice. A smaller number of bacteria apparently was ineffective. It is probable that, inasmuch as the twenty mice employed for this test were obtained from the breeding room, they had been previously exposed to the infection. As already stated, recovery from the disease induces resistance to the effects of a later inoculation of the organism.

When the cultures were introduced intraorally by means of a syringe fitted with a blunt needle into four quarantined mothers, seven of twenty in the litters they were then suckling developed the non-lethal experimental disease. As a control, two mothers were treated similarly with paracolon bacilli with no evidence of illness in their litters; whereas two mothers given *Salmonella aertrycke* cultures *per os* showed in their litters of twelve young, characteristic mouse typhoid in all of them with a mortality of 80 per cent.

Possible Toxin Production.—Intraperitoneal inoculation of 0.5 cc. of a Seitz filtrate of a 48 hour broth culture resulted in non-lethal enteritis in two of seven infant mice. Since the filtrate was free from microorganisms there is a possibility that the bacterium produces a toxin, but this substance is either weak or small in amount.

The pathogenicity of the organism is of a lower grade than that of the *enteritidis-aertrycke* or mouse typhoid organisms (24, 25). This is substantiated by the apparent insusceptibility of adult mice, the lower mortality and morbidity among artificially infected young mice, and the low power of invasion in the natural disease. Infection with a mouse typhoid organism is followed by a septicemia that results in macroscopic changes in the liver and spleen with the infecting organism readily recoverable from these organs. In this epizootic it was exceptional to recover the organism from the blood, liver, and spleen in the natural infection.

Carriers

The production of the disease in infant mice following intraoral administration of the bacterial cultures to mothers led us to consider the possibility of conveyance of infection by way of the mother's milk. However, in six experiments we failed to recover the organism from the milk.

In the first three recovered mice which were cultured, the Salmonella organism was obtained in each instance from intestinal contents. Hence one may infer that carriers exist and that the source of infection is in fecal, rather than milk, contamination.

DISCUSSION AND SUMMARY

It is now quite generally conceded that the presence in animals of bacteria of Salmonella type is indicative of an active, or potentially active, pathogenic agent (26). In the present instance bacteriological studies of the organs of acutely ill infant mice revealed the presence of one of the Salmonella group. It showed itself to be such by an absence of fermentation of lactose and saccharose, by other cultural reactions, and by cross-agglutination with certain members of the Salmonella group in very low dilutions of their antisera.[6] However, the bacterium cannot be included in any of the known species of the group because of its indol-forming properties, differences in carbohydrate reactions, and specific serological reactions. Its presence in affected mice, growth on artificial media, ability to cause the disease following enteral and parenteral inoculations, and the fact that it can be recovered from artificially infected mice fulfill the postulates of Koch. Furthermore, agglutinins are present in the blood of recovered mice and not in that of normal animals, and recovery of mice from natural infection evokes an apparent resistance against the special recovered Salmonella bacterium. The organism would appear to fall in the *Asiaticus* division of the genus, as designated by Castellani and Chalmers (27).

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[6] The organisms *Salmonella enteritidis* Gaertner, *aertrycke* (two strains), *paratyphi*, *schottmülleri* Seiffert (two strains), and *suipestifer*; and *Eberthella typhi*; *Shigella dysenteriae* Shiga, and *paradysenteriae*, variety Flexner, were used in the preparation of antisera. With the organism recovered in our studies cross-agglutination was present in titers not exceeding 1:200 only in the case of *Salmonella enteritidis*, *aertrycke*, *schottmülleri* Seiffert, and *suipestifer* antisera.

CONCLUSION

A study is herewith reported of an epizootic limited in its occurrence to suckling and newly weaned mice. The primary causal agent appears to be a bacterium of Salmonella type.

BIBLIOGRAPHY

1. Flexner, S., *J. Exp. Med.*, 1922, **36**, 9.
2. Lynch, C. J., *J. Exp. Med.*, 1922, **36**, 15.
3. Amoss, H. L., *J. Exp. Med.*, 1922, **36**, 25.
4. Amoss, H. L., *J. Exp. Med.*, 1922, **36**, 45.
5. Webster, L. T., *J. Exp. Med.*, 1922, **36**, 71.
6. For the extensive bibliography of L. T. Webster, see his general article in *Harvey Lectures*, 1931–32, **27**, 154.
7. Amoss, H. L., and Haselbauer, P. P., *J. Exp. Med.*, 1922, **36**, 107.
8. For a review of the subject see editorial, *J. Comp. Path. and Therap.*, edited by Sir John M'Fadyean, 1933, **46**, 257.
9. Salmon, D. E., and Smith, Theobald, *U. S. Dept. Agric., 2nd Ann. Rep. Bureau Animal Ind.*, 1885, 212. Welch, W. H., *Bull. Johns Hopkins Hosp.*, 1889–90, **1**, 9.
10. Edwards, P. R., *J. Infect. Dis.*, 1934, **54**, 85.
11. Rettger, L. F., and Harvey, S. C., *J. Med. Research*, 1908, **18**, 277.
12. Rettger, L. F., *J. Med. Research*, 1909, **21**, 115.
13. Rettger, L. F., and Scoville, M. M., *J. Infect. Dis.*, 1920, **26**, 217.
14. White, P. B., in A system of bacteriology in relation to medicine, Great Britain Medical Research Council, London, His Majesty's Stationery Office, 1929, **4**, 86.
15. Pritchett, I. W., *J. Exp. Med.*, 1925, **41**, 209.
16. Olitsky, P. K., Syverton, J. T., and Tyler, J. R., *J. Exp. Med.*, 1933, **57**, 871.
17. Topley, W. W. C., *J. Hyg.*, Cambridge, Eng., 1920–21, **19**, 350.
18. Topley, W. W. C., Weir, H. B., and Wilson, G. S., *J. Hyg.*, Cambridge, Eng., 1921, **20**, 227.
19. Andrewes, F. W., *J. Path. and Bact.*, 1922, **25**, 505.
20. Webster, L. T., *J. Exp. Med.*, 1922, **36**, 97.
21. Webster, L. T., *J. Exp. Med.*, 1923, **37**, 231.
22. Topley, W. W. C., and Ayrton, J., *J. Hyg.*, Cambridge, Eng., 1924–25, **23**, 198.
23. Topley, W. W. C., Ayrton, J., and Lewis, E. R., *J. Hyg.*, Cambridge, Eng., 1924–25, **23**, 223.

24. Webster, L. T., *J. Exp. Med.*, 1923, **38**, 33.
25. Webster, L. T., *J. Exp. Med.*, 1923, **38**, 45.
26. Lovell, R., *J. Comp. Path. and Therap.*, 1932, **45**, 27.
27. Castellani, A., and Chalmers, A. J., Manual of tropical medicine, London, Ballière, Tindall, and Cox, 3rd edition, 1919, 939–940.

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RABBIT POX

I. CLINICAL MANIFESTATIONS AND COURSE OF DISEASE

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PLATES 19 TO 21

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In December, 1932, a disease presenting many of the characteristic features of small pox in man appeared in a colony of rabbits maintained for the study of problems of constitution and quickly assumed epidemic proportions with a high mortality in certain classes of animals. The nature of the disease and the origin of the outbreak were obscure, but the picture presented indicated that the disease was due to a virus and not to a bacterial infection. This impression was supported by observations made in the spring of 1930 when there was a small epidemic of a disease presenting the same clinical and pathological picture.

While it is known that infection with vaccine virus may be transmitted from one rabbit to another by contact or to other animals in the same room, the prevailing impression is that these infections usually are mild or asymptomatic. However, a number of severe epidemic infections have been attributed to neurovaccine virus. In the present instance, work with a number of viruses was being carried on in several laboratories, but at the time very little information could be obtained concerning the use of vaccine virus. It was subsequently learned, however, that prior to the outbreak of the epidemic in question, at least three laboratories had inoculated rabbits with vaccine virus and that in two of these neurovaccine had been used. Some of the inoculated animals were on a lower floor of a building in which a small section of the breeding colony was kept, while others were on the same floor of the building in which the main colony was housed and there were connecting links by means of which a highly contagious infection might be transported from one group of animals to another, either in the same or in different buildings. If dissemination of vaccine virus occurred among the experimental animals in these laboratories, it apparently was not detected before the outbreak of this highly contagious disease in the breeding colony.

It seemed desirable to investigate this disease as fully as circumstances would permit. The colony in which the epidemic occurred is composed of pure bred and hybrid stocks of many breeds, and at the time of the outbreak contained more than 1,400 rabbits. Some were normal vigorous animals bred to standard specifications, while others showed or were known to transmit various physical and functional abnormalities. The colony had been under systematic observation for several years. The pedigrees and life histories of all animals were known, and the racial, familial and individual peculiarities of the stock had been made the subject of extensive investigation so that an unusual opportunity was presented for the study of the epidemic from several points of view.

Clinical, pathological and epidemiological studies of the spontaneous disease were carried out by the author; etiological studies were made by Dr. Pearce, Dr. Rosahn and Dr. Hu. All of this work was restricted by the necessity for the preservation of as much of the colony as possible, the use of measures to prevent the spread of the disease within the colony as well as to other stocks of rabbits, and by the lack of adequate facilities for the systematic management and study of an epidemic of such extensive proportions. Despite these handicaps, a fairly complete series of observations and experiments was carried out.

Investigations showed that the disease had been present in other stocks of rabbits for some time before it appeared in our breeding colony; later it was found in still other stocks, including animals under experimental observation, normal animals in the general stock room from which animals are obtained for experimental purposes, and in the Institute breeding colony. It is evident, therefore, that information concerning this disease is not only of medical interest, but is also of interest to those who breed rabbits or use them for experimental purposes. The reports to be submitted are intended to cover these several fields of interest, but the spontaneous disease and the epidemic have been studied with especial reference to the rôle of predisposing and constitutional factors for which the material is peculiarly appropriate.

A brief summary of the outstanding features of the disease has been published.[1] The purpose of this paper is to present the clinical studies in more complete form. This will be followed by papers dealing with the pathology of the disease and an analysis of the results obtained from a study of the epidemic. Experiments dealing with the transmission of the infection, the disease produced by inoculation and the identification of the infectious agent will be reported by Pearce, Rosahn and Hu.

Materials and Methods

Study of the spontaneous disease in its several relations was based on a stock of between 1,400 and 1,500 rabbits. A detailed classification of these animals will be given in connection with the epidemiological studies. For present purposes, it is sufficient to say that the colony contained pure bred and hybrid stock from sixteen breeds including males and females ranging in age from several years to new-born young. There were males in active service and others in reserve, while the females included resting, pregnant and nursing does. There were young of all ages and many litters were born during the epidemic.

In the routine conduct of the colony, every litter is examined at birth and a record made. This is followed by a daily inspection of all young and of all nursing does for the purpose of noting any unusual development or any condition which requires attention, and the entire colony is examined once a week. These observations are made by members of the staff, but in addition the caretakers have been trained to note and report anything unusual, thus affording a double check on the condition of the colony from day to day.

Early in December the colony showed evidence of deterioration. Breeding operations were suspended, and a careful search was made for cases of illness including this particular disease. It was 2 weeks, however, before the first case was found. From this time on, cages containing infected animals were noted and all possible precautions were taken to prevent spread of infection until the disease became so widespread that further precautions appeared to be useless. However, strict isolation of the colony was maintained for about a month after the last case of the disease developed and all external lesions had healed.

During the epidemic, dead animals were collected daily for record and autopsy, cages containing young animals were inspected and obvious new cases of illness were recorded; sections of the colony were examined carefully day by day, especial attention being devoted to those animals which hitherto had shown no signs of illness. In this way, the entire colony was covered about twice a week and we were able to determine with reasonable certainty which animals, if any, had escaped infection. In the handling of supposedly uninfected animals, hand disinfection was carried out.

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[1] Greene, H. S. N., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 892.

As a part of the routine management of the colony, all dead animals are autopsied. During the epidemic a special effort was made to distinguish between deaths due to rabbit pox and deaths due to other causes, to note conditions peculiar to rabbit pox and complications associated with the disease. The pathological material obtained in this way was supplemented by material from animals killed during all stages of the infection, including the period of recovery, and by material from animals presenting complications and sequelae of various kinds, especially those which persisted or developed after apparent recovery from the pox infection.

Experiments were carried out to determine the length of the incubation period, the probability of acquiring the infection by the contact of normal with recovered animals, by the mating of normal with recovered animals and by offspring from recovered parents. Experimental tests were also made to determine the extent of the effect produced on the reproduction capacities of both males and females.

Signs and Symptoms of Disease

The disease designated as rabbit pox occurred in two distinct forms, the one an asymptomatic affection which was recognizable only by the presence of characteristic lesions, while the other presented definite symptoms of an acute infection. It was found, however, that in either case the diagnosis could not be made with certainty except by careful examination and the detection of certain distinctive lesions. The lesions found on examination of living animals are divisible into four groups: (1) lymphadenitis, (2) papular lesions of the skin and mucous membranes, (3) keratitis or ophthalmia and (4) orchitis. These will be described in the order given.

Lymphadenitis.—The lymphoid system was invariably affected and usually the first definite sign of infection was enlargement and induration of the popliteal lymph nodes. The nodes of the inguinal region and anterior triangle of the neck were also consistently enlarged, and the lymphoid masses of the pharynx were usually swollen and in many cases filled the greater part of its posterior portion. In other regions, enlarged nodes were occasionally found but, as a rule, no appreciable change could be detected by palpation. Lymphadenitis persisted throughout the disease and in some animals was the only sign of infection.

Lesions of Skin and Mucous Membranes.—A macular rash preceded the appearance of papules and occurred either as the first sign of infection or a day or so after the discovery of enlarged popliteal lymph nodes. The rash was visible on those parts of the body not covered by hair and could be demonstrated elsewhere by clipping or shaving.

After a variable time papules erupted in the macular areas (Figs. 3 and 4). They first appeared as extremely small elevated swellings and in some cases remained hardly visible or palpable throughout the infection. In other cases, growth occurred in an irregular manner and all variations in size up to nodules a centimeter in diameter were present in the same animal. They eventually became umbilicated, dry and covered with crusts. The vesiculation and pustule formation characteristic of small pox did not occur. Crops of macules and papules appeared in successon, and all stages of papular development could be found in a single area as in chicken pox. In severe infections, there were hemorrhages in the skin and papules tended to be hemorrhagic.

Papules were usually scattered irregularly through the skin of the entire body and were detectable with the greatest ease in the ears (Figs. 5 to 7), the lips, the eyelids and brows, the nape of the neck, the skin of the trunk and the scrotum (Fig. 17). Occasionally they occurred as confluent masses, but more commonly, although arranged in concentrated groups, individual lesions remained discrete. Such distributions were usually associated with an abnormal looseness of the coat and affected areas could be plucked bare with little effort.

Papules were also common on mucous membranes and often occurred in confluent masses associated with extensive edema. They were found on the lips and inner surface of the cheek in most cases of severe infection (Fig. 9), and when confluent presented a striking picture. The edema usually involved neighboring tissues and caused an intense swelling (Fig. 10) which, in some cases, became hard and indurated and persisted long after other signs of infection had disappeared. Such areas often became necrotic and sloughed with the production of large defects which healed with scar formation and resulted in permanent disfigurement (Figs. 19 and 20). The tongue and palate were frequently the seat of hard nodular infiltrations (Fig. 15), the centers of which often became necrotic and sloughed with the formation of deep ulcers. Occasionally the gums showed a diffuse greyish swelling with localized areas of necrosis extending to the roots of the teeth, which caused permanent damage or loss of teeth in recovered animals. These lesions were usually accompanied by a clear blood-stained secretion or a mucopurulent discharge from the nose, suggestive of snuffles (Fig. 8), which dried with the formation of thick, brownish, adherent crusts.

Papules were also found about the anus, on the vulva of the female and on the sheath in the male. In these situations they usually occurred as discrete lesions. Occasionally they were confluent and accompanied by edematous swelling which caused obstruction of the urethra and retention of urine, and sometimes resulted in necrosis of the parts.

An extensive edema of ears, legs, genitalia and tissues about the eye was also of common occurrence when such parts were severely affected.

Eye Lesions.—Eye involvement was of almost constant occurrence and resulted in a variety of lesions (Figs. 11 to 14). Many cases of infection showed only a slight marginal blepharitis with excessive lacrimation, which usually accompanied the macular rash but sometimes preceded it. In some cases, infection spread to the lacrymal duct and a chronic dacryocystitis resulted. Diffuse keratitis with corneal ulceration was common. Iritis and iridocyclitis occurred later in the course of infection and were often followed by secondary glaucoma. In many animals, the occurrence of those individual lesions was masked by an outspoken purulent ophthalmia. Typical eye lesions were occasionally the only visible sign of infection.

Orchitis.—In males, the testicles usually showed a nodular or diffuse orchitis with edema of the scrotum (Figs. 16, 17). The involvement in many cases was so extensive that sterility of animals seemed inevitable. Lesions resolved, however, with restoration of fertility in most instances.

Clinical Course of Disease

Incubation.—During the epidemic, it was impossible to fix the time of exposure of a given animal or of animals in a given cage, so that the incubation period could not be determined with accuracy. However, observations made on cage groups showed that there was considerable variation in the interval between the development of the first and subsequent cases of infection in the same cage. During the early stages and at the height of the epidemic, there was rarely more than 2 to 3 days between the development of the first and last cases, and not infrequently all of the animals came down at the same time. As the epidemic progressed, the irregularity increased until, in the terminal stages, the interval between known exposure and the appearance of definite lesions was frequently as much as a week and occasionally as long as 2 weeks.

In order to determine the incubation period with greater accuracy, two experiments were carried out during the terminal stages of the epidemic. In each case, six rabbits 2 months old and of the same litter were exposed to infection in the following manner. In one experiment the six rabbits were placed in a clean cage with a rabbit of about the same age which showed an active infection, and left for 24 hours after which the infected animal was removed. In the second experiment the animals were placed in a cage of the same size in which all except one of a group had died. The survivor was in the terminal stages of the disease and died in less than 12 hours.

There was no decided difference between the results of the two experiments. Most of the animals came down between the 5th and 7th days after exposure. The shortest incubation period was about 4½ days and the longest 9 days. It seems probable that during the height of the epidemic the average incubation period may have been shorter. On the other hand, it is known that, during the terminal stages, the incubation was as long as 2 weeks in some instances, and may have been longer.

Symptomatology.—Constitutional symptoms of infection varied both in occurrence and severity, dependent upon breed, age and physiological status of affected animals, and, as has been pointed out, were frequently absent. A full account of this variation will be found in the report dealing with epidemiological studies.

In typical cases the onset was marked by an elevation of temperature which in some instances was as high as 106°F., and animals were obviously ill. Apathy and loss of appetite were early symptoms and rapid prostration was not uncommon. Diarrhea was common in the early part of the epidemic, both in young animals and in adults, but was infrequent later and its occurrence in the early stages of the epidemic was probably influenced by a coexisting dietary disturbance.

Symptoms resulting from involvement of individual organs were more constant. Infection of the upper respiratory tract was accompanied by labored respiration, mouth breathing and extreme weakness. Photophobia was commonly associated with lesions of the eye and could be recognized by partial closure of the lids. Animals with extensive lesions of the mouth and tongue generally refused food, probably more because of the pain caused by eating than from actual loss of appetite, and rapidly became emaciated.

Nervous system involvement was manifest in some animals by incoordinate muscular movement, fibrillary twitching and nystagmus. Paralysis occurred in other instances and was usually limited to special muscle groups. The sphincter muscles were most commonly affected, and such cases showed dribbling of urine and frequent passage of loose fecal material which adhered to the perineum, hardened and, unless removed, blocked the anus and urethra, causing retention of urine and feces. Generalized paralysis was not common and usually occurred in the terminal stages of a severe infection.

Types of Disease.—The majority of affected animals showed generalized lesions and symptoms referable both to systemic infection and involvement of individual organs. In many instances, however, lesions were more prevalent or conspicuous in a particular organ or part and although strict localization seldom occurred, this inequality allowed a basis for recognition of other disease types (Figs. 1, 2). Thus, in some cases the eruption in skin and mucous membranes constituted the clinical picture, while in others glandular enlargement, orchitis or ophthalmia was the only external manifestation of infection.

The limitation in distribution did not affect the severity of lesions, for they were no more pronounced than when associated with lesions of other parts. A purulent ophthalmia often persisted in an animal as the only sign of infection. On the other hand, a slight inflammation of the lids without lesions elsewhere was of equally frequent occurrence.

Symptoms of constitutional disturbance bore little relationship either to distribution or severity of external lesions. The mildest cases of ophthalmic, genital and cutaneous infections were usually asymptomatic, but occasionally showed the severest signs of ill health. Moreover, animals with extensive lesions of these parts often appeared in perfect health. Asymptomatic cases were also common both in animals with widespread lesions and in others in which lesions were so poorly defined that their presence could only be detected by the most careful examination. In respect to mortality, however, such cases were as serious as others, and at autopsy animals showed typical changes in internal organs.

Mild and abortive types of infection were common during the terminal stages of the epidemic, and a large proportion of animals of the most susceptible breeds and age groups survived infection contracted at that time.

Effects in Pregnant and Lactating Does.—In pregnant and lactating does, infection produced the severest symptoms.

Such animals were extremely ill and presented the characteristic facies of the disease, hunched in a corner of the cage in a stupor, breathing stertorously through nostrils obstructed by thick brownish crusts, lids partially closed over dull, lusterless eyes or glued together by dried secretions and, when forced apart, revealing a diffuse keratitis or destructive ophthalmia. Pregnancy was commonly terminated by abortion and marked improvement usually followed. Abortion was also frequent in non-infected, apparently healthy does, both in the latter part of the pre-epidemic period and during the epidemic. Young born at term were usually either dead, abnormal in some respect and non-viable, or deserted by the doe. Clinical or pathological signs of infection were never found in feti or young born dead. Lactating does were even more seriously affected and unless relieved of the strain of nursing, rapidly deteriorated and died. The separation of young from nursing does often improved their condition and, in many instances, brought about rapid recovery.

Complications.—Some complications were of such frequent occurrence and so intimately associated with the disease that they have been described as signs of infection. Thus, corneal ulceration occurred in most animals showing eye involvement and was often followed by perforation, iridocyclitis or glaucoma. Chronic inflammation of the lacrymal duct with mucocele was occasionally observed. Laryngitis was a constant complication of upper respiratory infections and was frequently accompanied by edema of the glottis which caused the stertorous breathing characteristic of many cases. Infection of the submaxillary glands was common and occasionally resulted in visible enlargement.

In many cases death was apparently due to a terminal bronchopneumonia which was a frequent complication and in some instances may have resulted from the aspiration of foreign material due to diminished sensibility of the larynx. Pulmonary abscess was less common but was the direct cause of the majority of postepidemic deaths. Other secondary pyogenic infections were rare. Dental abscess occurred in a number of animals and in a few instances involved the bone of the jaw.

During the early part of the epidemic the disease was frequently complicated by gastrointestinal disorders. Diarrhea has been mentioned. In young animals, the discharges consisted of thick tenacious mucous but in adults were usually made up of loose watery fecal material. Intestinal obstruction caused by impactions in the cecal region was also common. These disorders were of frequent occurrence previous to the epidemic and are not attributable to the disease. However, they were serious complications and when superimposed upon the infection, usually resulted in death.

Mortality.—Death might occur within a few hours after infection was first noted or be delayed for days or weeks. The total mortality was 46.4 per cent but showed wide variations in different age groups and breeds. Among animals less than 14 weeks of age, 71.8 per cent of cases of infection died; the mortality rate was highest among those 4 to 8 weeks old. In adults, although the mean mortality was about 15 per cent, the rate in different breeds varied widely, certain lines being almost completely wiped out while in others no deaths occurred.

A full discussion of the mortality will be reserved for the report concerned with the epidemiological features of the disease.

Recovery.—Many animals with severe infections recovered (Fig. 18).

Prognosis based on the nature of symptoms and type of disease sometimes proved correct, but as a rule no indication of the final outcome of infection could be obtained from clinical examination.

In cases of recovery, lesions underwent slow resolution extending over a period of weeks, and in some instances healed with the formation of scars. Reactivation and reinduration of skin papules with the development of new lesions was occasionally noted after healing was well advanced, but it is not certain that relapse occurred.

Sequelae.—All recovered animals bear stigmata of the disease. Corneal scars and scarred hairless areas in the skin of the ears (Fig. 21) have persisted to the present time. Many animals were permanently disfigured by the healing of large defects by secondary intention and subsequent contracture (Figs. 19, 20). In other instances, the sloughing of portions of the genitalia rendered the animals unfit for breeding service.

A variety of abnormalities of the incisor teeth resulted from gum lesions and dental necrosis. In many animals both primary and secondary incisors were completely destroyed, and in others regeneration resulted in malalignment which led to direct antagonism with loss of cutting edge or to an outspoken deformity in which antagonism was entirely lost and growth, therefore, unrestrained.

Although to outward appearances animals were sufficiently recovered and fit for the resumption of breeding 2 months after the beginning of the epidemic, the occurrence of two widespread disorders later in the spring indicated that not even at that late date had they reached a normal level of general health and vigor. Although a thorough investigation of these conditions could not be made, they appeared to be attributable in part at least to the reaction of convalescent and constitutionally weak animals to adverse nutritional or other environmental factors and, in this sense, were sequelae of the pox epidemic.

The first disorder to appear was characterized by jaundice, progressive weakness and emaciation. After prolonged illness most adults improved or recovered, but the disease was usually fatal in young rabbits. Those that died were autopsied and showed marked cirrhosis of the liver of mixed type, entirely different from the cirrhosis due to coccidiosis. The second condition was characterized by paralysis of the hind quarters, atrophy and degeneration of the fibers of the masseter muscles and extreme weakness—a condition resembling that attributed to the addition of excessive amounts of cod liver oil to the diet of rabbits and other herbivora. The two conditions were occasionally combined in a single animal and produced an affection which resembled clinically the hepatolenticular degeneration described by Wilson in man. The paralytic condition was the more fatal of the two and many seriously affected animals were killed. In addition to the muscular atrophy and degeneration, the only constant finding at autopsy was enlargement and cystic degeneration of internal lymph nodes. Gross changes in the cord or lenticular nucleus were not found and sections have as yet revealed no microscopic lesion.

The frequency of litter, cage and location occurrence of these diseases suggested the possibility of infection, but attempts to transmit the diseases gave negative results (Pearce, Rosahn and Hu). The animals were on a diet which contained cod liver oil, and it is possible that this may have contributed to the production of one or both of these conditions. But on other occasions much larger quantities of cod liver oil have been used with no ill effects. The cause of the diseases is still obscure. It is possible, however, that prolonged feeding of oil which may have been of poor quality or added in excessive amounts played some part in the occurrence of these affections.

Effects on Reproduction

Breeding was resumed about 2 months after the beginning of the epidemic and a study was made of the reproductive capacities of recovered animals. A number of normal animals were added to the colony and their breeding record was used as a control. After a preliminary low fertility rate, the proportion of pregnant matings in recovered stocks rose rapidly, equalled that in normal animals, and by the end of April the unprecedented figure of 100 per cent fertility was reached. Abortions, still births and desertions were no more frequent than under usual conditions.

In males testicular lesions resolved, parenchyma regenerated and the fertility of all recovered animals has been proved. Occlusion of the Fallopian tubes in the female occurred in some cases and sterility resulted. In most instances of this kind, however, blockage was present in only one horn of the uterus and the other bore young in a normal fashion.

During the fall following the epidemic, the fertility of the colony, which was made up almost entirely of recovered animals and their offspring, was higher than at any period previous to the outbreak. However, there was an increase in the number of deaths of pregnant and parturient females, and at autopsy all showed similar lesions consisting chiefly of changes in the liver comparable with those found in the toxemias of pregnancy. It is noteworthy that these were all recovered animals and showed large, persistent scars in the liver, but the exact relationship of the disorder to previous pox infection is uncertain.

Carrier Transmission

Following the epidemic many seriously affected animals were discarded. However, a selected group was retained for tests of carrier transmission which were performed simultaneously with tests of reproductive functions. Normal animals were mated with recovered animals, recovered males with normal females and recovered females with normal males, but in no instance was a mating of this kind followed by the development of disease. As a further test, normal animals of the most susceptible age groups were imported into the colony and were kept in continuous contact with recovered animals but showed no sign of infection. Moreover, approximately 500 litters containing over 2,500 animals have been born from recovered animals since the end of the epidemic. All of these young have been examined clinically and many have been subjected to postmortem examination, but in no case has any indication of the transmission of the infection been found.

Contagiousness of Infection

A comparison of the present epidemic with that of 1930 indicates that the contagiousness of the infection may vary greatly in different epidemics. In 1930 only a small part of the colony contracted the disease. Spread of the infection was effectually checked by prompt isolation or killing of animals which developed the disease, as was indicated by the fact that there were no immunes among the survivors of the first epidemic. In the present instance, however, isolation of rooms with systematic disinfection and removal of infected or apparently healthy animals to other quarters had no appreciable effect on the spread of disease.

At the beginning of the outbreak, infection invariably followed exposure and spread was so rapid that within 2 weeks the distribution was pandemic, and in less than a month nearly every animal in the colony had been infected. Toward the end of the epidemic there was, however, a decrease in both the contagiousness and severity of the infection so that a few litters born after the peak of the epidemic was passed entirely escaped infection; others showed only a mild disease with slow spread of the infection. Moreover, two experimental groups of the most susceptible age introduced into the colony at that time survived the disease.

The pathology of epidemic rabbit pox will be described in a separate paper and the clinical manifestations will be discussed in conjunction with this phase of the subject.

SUMMARY

The clinical manifestations and course of a highly contagious and acutely fatal disease designated as rabbit pox have been described. The general symptomatology of this infection varies within wide limits and accurate diagnosis rests on the occurrence of certain characteristic lesions. The most distinctive sign of infection is a pock-like eruption which is often outspoken and widespread over the body, but is sometimes poorly defined and detectable only after careful physical examination. Other lesions depend upon the involvement of particular organs and give rise to special symptoms. As a rule, the incubation period of the epidemic infection varies between 5 and 7 days. No evidence of the transmission of the infection by recovered animals could be obtained. Furthermore, breeding tests demonstrated that the reproductive capacities of most animals were not permanently impaired.

EXPLANATION OF PLATES

PLATE 19

FIGS. 1 and 2. Litter mates with different types of disease. The course of infection was mild in the animal shown in Fig. 1 and external lesions were limited in the skin, while the animal in Fig. 2 was seriously ill and its posture is a manifestation of acute respiratory distress resulting from extensive mouth lesions.

FIG. 3. Cutaneous lesions on the trunk of a young hybrid animal. The coat in this area was abnormally loose and was easily plucked by hand. Individual papules show considerable variation in size; many approach a centimeter in diameter, while others are barely visible. This difference is not entirely due to the

age of the lesion, for marked variation in size is also found among papules of equal maturity. Many of these lesions are hemorrhagic.

FIG. 4. Skin of a pregnant Himalayan doe self-plucked for nest fur, showing both dry, crusted papules and others in earlier stages of development. Minute lesions of this type occurred in all adult Himalayans.

PLATE 20

FIG. 5. Transilluminated ear showing early macular lesions distributed in the more vascular areas.

FIG. 6. Ear at the height of infection showing typical papules.

FIG. 7. Ear showing unusually large papules with umbilication.

FIG. 8. Sanguinous discharge from the nares in an acutely fatal infection. This was a frequent early sign of disease, and in the young Belgian rabbit shown in this photograph it was the only external sign of infection.

FIG. 9. Typical papular lesions of the lips.

FIG. 10. Diffuse edematous infiltration of the muzzle in a young Rex rabbit. Compare with Figs. 8 and 9.

FIG. 11. Eye showing diffuse clouding of the cornea. This was the only visible lesion found in this animal—a monosymptomatic infection with no constitutional symptoms.

FIG. 12. Panophthalmia with total destruction of the lens.

FIG. 13. Purulent ophthalmia with ectropion of lids.

FIG. 14. Unilateral secondary glaucoma. A common sequel of ophthalmic infections.

FIG. 15. Nodular lesions of the tongue and palate with infiltration of gum margins.

PLATE 21

FIG. 16. Diffuse orchitis with edema of scrotum in an adult Belgian buck.

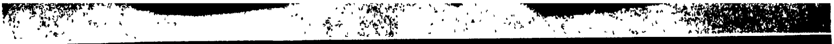
FIG. 17. Nodular orchitis with papular lesions of scrotum in an American blue buck.

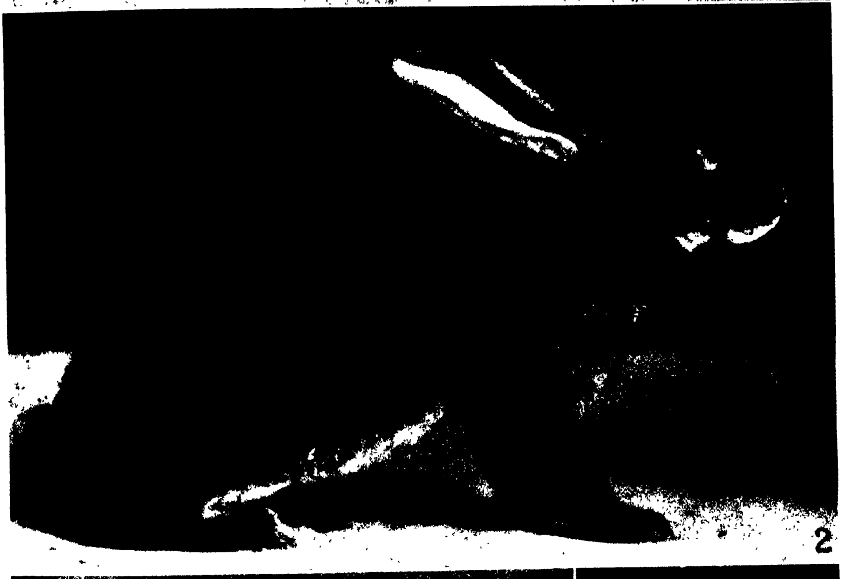
FIG. 18. Recovery with permanent impairment of vitality after severe illness.

FIG. 19. Healing of a diffuse lesion with contracture resulting from necrosis and sloughing of a portion of the right upper lip.

FIG. 20. Gangrene followed by sloughing of upper lips and nose in a young Rex. The incisor teeth show malocclusion resulting from the infection in which the upper and lower cutting edges are in apposition. Root infections with permanent injury to the teeth were common.

FIG. 21. Scarred hairless areas in the skin of the ears 1 year after recovery.





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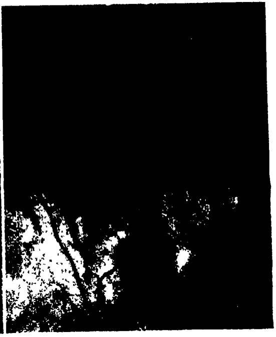

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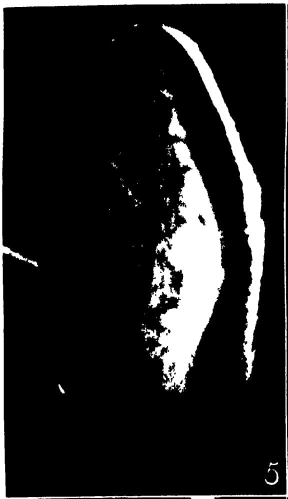
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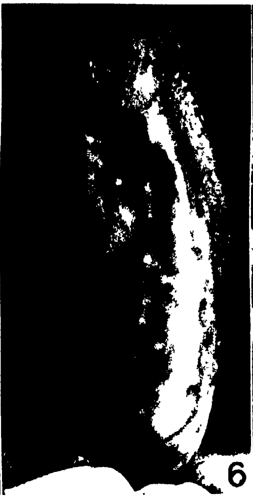
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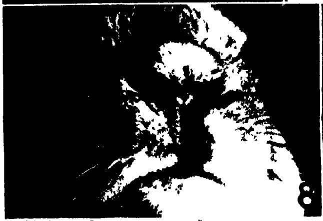
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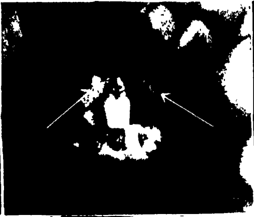

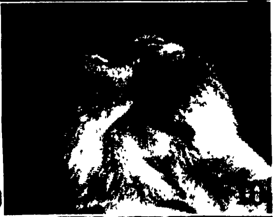
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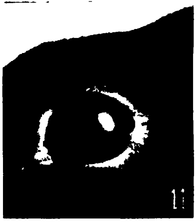
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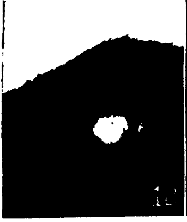
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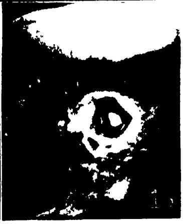
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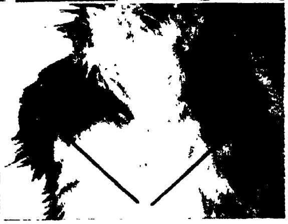

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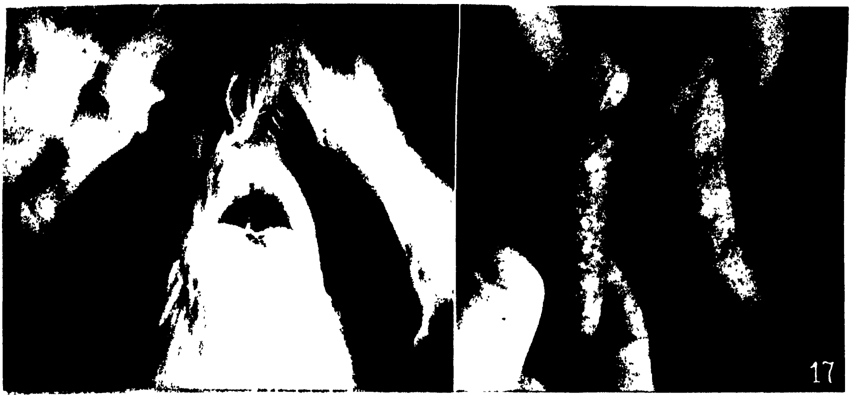

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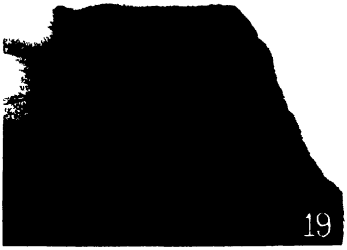

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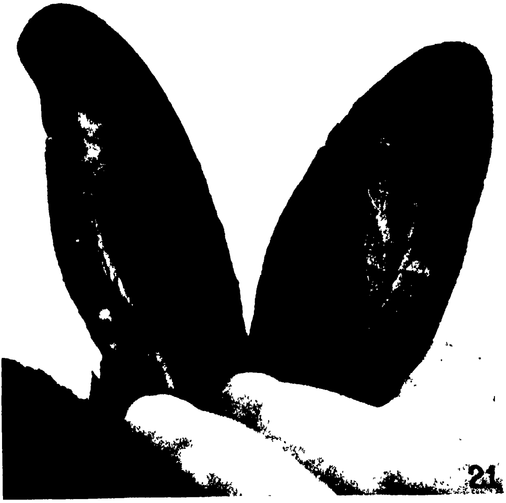

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(Greene: Rabbit pox. I)

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RABBIT POX

II. PATHOLOGY OF THE EPIDEMIC DISEASE

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PLATES 22 TO 24

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The clinical manifestations of epidemic rabbit pox have been described.[1] The purpose of this paper is to record the results of pathological examinations.

Material and Methods

The epidemic of rabbit pox was preceded by an increase in gastrointestinal and nutritive disorders and infections of the upper respiratory tract with a consequent increase in the number of deaths due to those causes. These conditions were prevalent during the epidemic so that animals coming to autopsy represented not only deaths due to rabbit pox, but also deaths due to other causes and to rabbit pox complicated by other conditions.

During the epidemic, postmortem examinations were made on over 900 rabbits. These included deaths from various causes and animals killed for the purpose of obtaining material representing all stages of the disease uncomplicated by other conditions. The methods employed in the collection of material were outlined in the previous paper. Tissues were fixed in 10 per cent formalin, Zenker's and Helly's fluids and sectioned in paraffin. Sections were stained with hematoxylin and eosin; Giemsa's stain was also used in examinations made for inclusion bodies.

Gross Postmortem Findings

The postmortem picture was not always characteristic and in some cases diagnosis was questionable, owing to the absence of distinctive macroscopic lesions or to the confusion created by other conditions prevalent at the time. As a rule, however, marked alteration in the gross appearance of tissues and organs and the occurrence of widespread focal lesions in the skin or elsewhere made identification of the disease possible.

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[1] Greene, H. S. N., *J. Exp. Med.*, 1934, 60, 427.

Areas of focal infiltration and necrosis were nearly always present and formed the most definite evidence of infection. Apart from the skin (Figs. 1, 2), mouth (Figs. 8, 9) and upper respiratory tract, they occurred with the greatest frequency in the liver, spleen and lungs, but were found in every organ of the body with the exception of the brain and kidneys.

The general appearance of animals dying from fulminating infections necessarily differed from that of animals in which the course of disease was more protracted. As a general rule, however, animals were well nourished with an abundance of fat in normal depots, and only in those cases associated with obstruction of the pharynx or with extensive ulceration of the tongue and mouth was extreme emaciation found.

Subcutaneous tissues were often markedly edematous and pea-sized nodules of necrosis were occasionally found in fat and connective tissue. Muscle and serous membranes rarely showed clearly defined focal lesions but small miliary hemorrhages were common (Fig. 3).

Lesions within the Abdominal Cavity

The peritoneal cavity usually contained an excess of free fluid which in rare cases was blood-stained. Small areas of fat necrosis were common in the omental, mesenteric and retroperitoneal fat, and occasionally characteristic pearly white nodules of pin-head size were scattered over the surface of the peritoneum.

Stomach and Intestine.—The stomach was usually filled with food, and in many cases, although autopsy quickly followed death, digestion and perforation of the walls had occurred. Small petechial hemorrhages were occasionally found on the surface of the cecum and small bowel, but otherwise focal lesions were extremely rare.

In some cases, the intestines were distended with fluid and gaseous material and the anal region stained with diarrheal discharges. In others the cecum and upper portion of the colon were filled with masses of fecal material which were hard and dry or of a stiff, putty-like consistency. These masses were of variable extent and usually they were separated by accumulations of a thick tenacious mucoid material. Occasionally, this condition was found in the lower portion of the small as well as in the large intestine. It was also present in animals dying of other causes before and during the epidemic so that it cannot be regarded as peculiar to rabbit pox. It is rather evidence of an abnormality connected with the epidemic and accentuated by pox infection.

Liver.—The liver was always involved and usually had a characteristic appearance. In typical cases it was moderately enlarged, firm in consistency and pale yellow or yellowish brown in color with a profusion of translucent pearly white or opaque grey nodules a millimeter or more in diameter scattered over its surface and distributed throughout the organ (Fig. 5); nodules were occasionally found in the wall of the gall bladder. In some instances, these small lesions were accompanied by larger irregular areas of necrosis measuring as much as 0.5 cm. in diameter. In other cases the liver was markedly enlarged and showed an extreme degree of yellowish red mottling suggestive of passive congestion. In fulminating cases, characteristic nodules were usually absent.

Spleen.—The spleen was invariably affected, and as a rule was moderately enlarged, dark red in color, firm and showed a few small areas of necrosis comparable with those found in the liver (Fig. 6).[2] The greatest enlargement occurred in fulminating cases and in those associated with secondary infection. Under these conditions the spleen was soft and considerable blood and pulp could be scraped from its cut surface. Exceptionally, the organ was normal in size, a dirty greyish brown in color and showed numerous focal lesions.

Kidneys and Adrenals.—The kidneys were enlarged and pale but characteristic macroscopic lesions were never demonstrated. The adrenals were usually large and greyish yellow in color; they were soft and showed a loss of architectural definition suggestive of autolysis, but occasionally distinct areas of focal necrosis were found.

Genitalia.—In the female the ovaries and uterus were often riddled with focal lesions. Infection of the uterus was frequently accompanied by a purulent exudate and abscesses were sometimes found in the wall. This was particularly true of animals which had recently aborted or given birth to young. The testicles of the male were always enlarged, and in most instances contained numerous focal and diffuse areas of necrosis varying from minute nodules to lesions a centimeter or more in diameter. Edema and hemorrhage were present in some cases.

Lesions within the Thorax

The pleural and pericardial cavities usually contained an excess of clear fluid and occasionally were markedly distended. Purulent exudates were not seen. Focal lesions were common on pleural surfaces but rare on the pericardium.

Lungs.—The lungs showed a variety of conditions. Visible or palpable lesions were nearly always present but occasionally the lungs were normal in appearance. The most constant lesion was a small subpleural nodule, and in some instances these were peppered thickly over all surfaces (Fig. 4). In early and uncomplicated infections, these translucent or pearly white nodules were frequently the only lesions present. More advanced cases showed typically necrotic, miliary nodules and occasionally larger pea-sized areas of necrosis with irregular patches of consolidation (Fig. 7). These areas of consolidation frequently measured a centimeter or more in diameter on the pleural surface and extended into the underlying tissue of the lung as a conical or wedge-shaped mass. They were greyish yellow or a dirty greenish grey color and sometimes were surrounded by a narrow zone of hemorrhage or congestion as in the case of an infarct. Occasionally these were the only macroscopic lesions found. Diffuse consolidation of one or more lobes was seen in some animals but was less frequent than other lesions.

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[2] The spleens shown in Figs. 5 and 6 are from Belgian hares. The enlargement is more marked than was generally observed at autopsy and is due in part to a coincident abnormality of the spleen found among animals of this breed in our colony.

The trachea and bronchi appeared normal in early cases. In more advanced cases, they usually contained an abundance of white frothy material which rarely was blood-stained.

Heart.—The heart was usually pale in color, soft and relaxed. In other instances it was normal in appearance. In a few cases marked distension of all chambers with fluid blood was noted. Rarely focal lesions were found in the myocardium.

Thymus.—The thymus was generally enlarged and occasionally markedly edematous. Petechial hemorrhages were of almost constant occurrence and focal areas of necrosis were common.

Lesions of Miscellaneous Organs

Lymph Nodes.—Lymph nodes were not universally affected. The most pronounced abnormalities were found in the pharyngeal and deep cervical nodes and in the superficial nodes in general, especially the popliteals. As a rule, these nodes were swollen and edematous and frequently showed characteristic focal lesions; occasionally they were dark red in color or contained petechial hemorrhages. In contrast with this, characteristic changes were rarely seen in the thoracic and abdominal nodes. Occasionally they were slightly swollen and edematous but, as a rule, no gross alterations could be detected.

Bone Marrow and Periosteum.—The bone marrow was always abundant but showed many variations in color and consistency. Frequently it was reddish grey in color with small scattered hemorrhages and larger greyish areas of necrosis. Under such conditions it was generally of normal consistency. In many instances it was uniformly grey in color and cheese-like in consistency. Rarely it was dark red and fluid.

The periosteum often showed greyish red elevated thickenings interspersed with areas of hemorrhage. These lesions were most common in the periosteum of the flat bones of the skull.

Thyroid and Parathyroids.—The thyroid, as a rule, was small and pale and often showed focal lesions. Parathyroids were likewise generally small but focal lesions were much less common.

Brain and Spinal Cord.—The brain ordinarily showed no other abnormality than an engorgement of pial vessels. In a few cases there was a slight increase in the amount of cerebrospinal fluid, and in one animal the ependyma of the lateral ventricles was markedly granular.

The spinal cord was usually normal in appearance. In a few instances it was slightly swollen and edematous.

Submaxillary Gland.—The submaxillary gland was almost invariably enlarged and contained localized and diffused areas of necrosis.

Microscopic Findings

Both in the gross and microscopically, the most distinctive lesion in all organs and tissues was the sharply circumscribed nodule or papule. More diffuse lesions arising either from the coalescing of adjoining nodules or the simultaneous affection of large areas were also common. Microscopically, the composition of these lesions varied somewhat with their location and age but, in general, affected areas showed comparable histological changes.

The fully developed nodule or papule was found to be made up of a central area of necrosis in which cell outlines and cell types were completely lost, surrounded by a zone of mononuclear cell infiltration. This zone and the neighboring tissues were usually edematous and occasionally hemorrhagic. In the more diffuse lesions the focal character was entirely lost, large areas of tissue were infiltrated, necrosis was widespread and edema was marked. Both types were found together in fatal infections, but among animals killed in early stages of the disease there was a notable tendency for the lesions of a given animal to be limited to either the discrete or the diffuse form.

Skin and Mucous Membrane.—Characteristic lesions of the various types were found in the corium and subcutaneous tissues. The epithelial changes characteristic of small pox in man were not found. In early lesions the epidermis remained intact and unaltered. Pathological changes were demonstrable only in those cases associated with large necrotic areas in the corium. These consisted in atrophy and degeneration and suggested secondary nutritional or pressure disturbances rather than the localization of the specific cause of the disease in the epidermis itself.[3]

Similar lesions were found in the mucous membranes of the mouth and pharynx but in this situation focal lesions were generally larger and diffuse edematous infiltrations were of more frequent occurrence.

A large amount of material from fatal cases of infection and from animals killed at earlier stages of the disease was examined with a view to determining the pathogenesis of focal and diffuse lesions. It was found that in both cases the lesions began as a vascular or perivascular process affecting blood vessels and lymphatics, but the development of the lesion was most clearly defined in the case of the smaller blood vessels. In the earliest lesions the endothelium of affected vessels was swollen and granular and occasionally detached. There was no apparent alteration in the walls of the vessel, but the perivascular tissues were slightly distended with a serous exudate and contained a few cells grouped in loose collar formation about the affected vessels. The cells were chiefly large mononuclears with some lymphocytes and occasional polymorphonuclear and red blood cells. There was also an appreciable tendency to the accumulation of leucocytes in the lumen of the vessel, and occasionally cells were seen penetrating the vessel wall.

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[3] In a recent epidemic of a disease presenting identical clinical signs and symptoms, the lesions of the skin were not limited to the corium, but were also found in the epidermis where they consisted of focal epithelial degeneration, vacuolization and vesicle formation. This disease was less contagious and far less fatal than that described in this report.

As the lesion progressed, the process assumed either a focal or diffuse form. In the case of focal lesions (Fig. 14), the accumulation of cells increased both inside and outside of the vessel. The walls of the vessel showed hyaline changes and eventually the outlines of the vessel were lost. The cells at the center of the nodule thus formed became necrotic and disintegrated so that cell outlines and cell types were no longer recognizable. At this stage, the lesion was composed of a central area of necrosis surrounded by a zone of mononuclear cells of variable extent. This zone and the adjacent tissues were edematous as a rule, and occasionally hemorrhagic.

Lesions of this type varied from extremely minute granules to masses a centimeter or more in diameter. They were widely scattered or in close proximity to one another but, as a rule, the intervening tissues were little if at all affected.

Diffuse lesions differed from the focal in that, from the beginning, there was a more widespread involvement of blood vessels and lymphatics with less tendency to the concentration of cellular elements about affected vessels. In typical cases, cells and fluid were spread diffusely through the tissues (Fig. 13); mononuclear cells still predominated but there was considerable increase in the relative number of polymorphonuclear and red blood cells. Widespread edema and extensive necrosis of tissues were characteristic features of these lesions and hemorrhage was a frequent occurrence.

There was no truly pustular stage in the development of either focal or diffuse lesions. With the extension of the necrosis in the deeper layers of the skin, the blood supply to adjacent tissues was cut off or impaired, giving rise to an anemic necrosis which frequently extended well beyond the limits of the specific lesion. In this way many focal lesions extended to the surface with the formation of ulcers covered by crusts, while confluent and diffuse lesions gave rise to mass necrosis or gangrene followed by sloughing of larger masses of tissue.

There was no evidence that a pyogenic or other secondary infection played any part in the production of these lesions. Focal and diffuse reactions had a common mode of origin, but from the beginning they pursued a different course. Evidently the difference was mainly one of degree and was referable to the extent of the vascular injury on the one hand, and the ability of the animal to limit the action of the causative agent on the other. As a rule, given animals showed a tendency to one type of reaction or the other, but with the progress of the disease focal lesions frequently became confluent and occasionally gave way to an outspoken diffuse reaction.

Lungs.—The lungs showed focal and diffuse lesions comparable in all respects with those of the skin. Circumscribed areas of mononuclear infiltration were found in the vicinity of small blood vessels (Figs. 12, 15) and occurred with the greatest frequency near the pleural surface of the lung, but were also situated deep in its substance and occasionally were scattered profusely throughout a section. They were rarely as sharply circumscribed as those found in the skin, and frequently alveolar walls extending from such foci were infiltrated and swollen with exudate. Occasional sections showed thickening and infiltration of alveolar walls with no clearly defined focal lesions. This infiltration was sometimes widespread but more often occurred in scattered patches without relation to the bronchi. More diffuse lesions in which large areas were infiltrated with an exudate rich in mononuclear cells and fluid and containing many polymorphonuclears were common in more advanced cases of infection, but were also found in association with early focal infiltrations. Older lesions showed varying degrees of degeneration, and in fatal cases total necrosis of an entire lobe was not uncommon. Lesions of this kind were comparable with the areas of mass necrosis seen in the skin. In rare instances, there were also extensive areas of consolidation composed mainly of polymorphonuclear cells which appeared to be due to the action of some secondary invader.

In early cases of infection the peribronchial lymphatic tissue was generally hyperplastic, but the bronchi themselves showed no pathological alteration. In many fatal cases, however, their walls were infiltrated and necrotic and their lumina plugged with exudate and desquamated epithelial cells. The absence of primary bronchial changes and the great frequency of focal interstitial lesions would suggest that the infection spread through blood and lymphatic channels rather than through bronchial passages.

Liver.—The liver was occasionally engorged with blood but, as a rule, showed in addition to the focal and diffuse areas of infiltration and necrosis only an extreme degree of cloudy swelling. Early foci were closely associated with blood vessels in the portal spaces (Fig. 10) but older and more diffuse lesions extended into the liver parenchyma and resulted in extensive destruction of lobules. Fatty degeneration was marked in some cases but was also a common finding in animals which showed no evidence of pox infection. In fulminating infections, typical focal necroses were not found and the microscopic picture was that of an acute diffuse degeneration and necrosis involving the whole organ. Individual cells were swollen, granular and indistinct with pyknotic nuclei, and in scattered areas were definitely necrotic. Mononuclear infiltrations were absent.

Spleen, Lymph Nodes and Bone Marrow.—The spleen showed a varying degree of congestion, which in some cases was extreme. The sinuses were distended and contained an excessive number of mononuclear cells; polymorphonuclear leucocytes were rare. Immature leucocytes and nucleated red cells were common. Malpighian corpuscles were generally enlarged, edematous and contained cells larger and more granular than normal; rarely the corpuscles were reduced in size. Focal and diffuse areas of necrosis were found in many instances. Focal lesions were most frequent on the surface but were also found in Malpighian corpuscles. Occasionally necrosis was widespread throughout the organ, the pulp consisting of a homogeneous mass of degenerating cells and pigment, while the Malpighian corpuscles were represented by nests of nuclear detritus.

Superficial lymph nodes were generally hyperplastic with large active germinal centers. Their increased size, however, was in large part due to edema. Sinuses were distended with fluid and individual cells widely separated. The deep cervical nodes were most markedly affected and frequently showed cystic dilatation of their medullary sinuses. These changes were often accompanied by discrete and diffuse areas of necrosis and, in many instances, entire nodes were necrotic.

The bone marrow in many fatal cases was involved in so widespread a necrosis that many sections failed to show a single normal cell (Fig. 11). In other instances small focal necroses were scattered throughout and hemorrhages were common. In less affected areas vessels were engorged with blood, large and small mononuclear cells were abundant, while polymorphonuclear cells were rare.

Miscellaneous Organs.—Cloudy swelling in the kidneys was so marked that many tubules were completely occluded. Other tubular or glomerular changes did not occur and necrotic foci were never found.

The adrenals often showed focal areas of necrosis. Widespread degeneration and disintegration were common in fatal cases but may have been of postmortem origin. In animals killed and immediately autopsied, however, similar but less marked changes were found. Diffuse areas of necrosis were of rare occurrence.

Discrete and diffuse areas of necrosis were common in the testicle. In early lesions there were scattered patches of mononuclear cells in the interstitial tissue and around the tubules. These increased and eventually became necrotic. In some instances, the necrosis was limited to small areas, while in others there was widespread necrosis of interstitial tissue and tubules.

The ovaries were usually hyperemic and frequently showed focal lesions. Simple cysts were also common. The walls of the uterus often contained multiple foci of necrosis and abscesses were occasionally found.

Necrotic foci, areas of hemorrhage and edema were frequent findings in the submaxillary gland, thyroid and thymus.

The walls of the gastrointestinal tract were generally hyperemic with small, scattered areas of hemorrhage and enlarged lymphoid follicles, but typical necrotic foci were not observed.

The heart showed no singular microscopic changes. Vacuoles were occasionally found in muscle fibers but cellular infiltrations or necrotic lesions were absent in the sections examined.

In other regions of the body muscle fibers were frequently separated by edema, and diffuse cellular infiltrations, hemorrhage and necrosis were common.

Many sections were taken from different parts of the brain, but aside from engorgement of vessels, no definite pathological changes were found.

Vascular Lesions.—Vascular damage was intimately associated with all lesions and formed the focus for the cellular infiltrations characteristic of the disease in all organs and tissues. Early changes consisting of swelling and desquamation of endothelial cells were found in small vessels and precapillaries in the vicinity of fresh infiltrations. In focal lesions the immediate surrounding tissues were infiltrated with mononuclear cells which, in some instances, could be observed migrating through the vessel wall. In diffuse lesions endothelial damage was more intense and more widespread. Later stages were characterized by hyaline degeneration and necrosis of the vessel wall. But in the larger vessels no distinctive lesion could be detected.

Inclusion Bodies.—A thorough search was made for inclusion bodies in specially cut and stained sections. Many tissues from animals in different stages of infection were examined, but in none were cytoplasmic or nuclear changes found which were sufficiently characteristic to be called inclusion bodies.

DISCUSSION

Epidemic rabbit pox is the most destructive contagious disease which we have encountered in the rabbit. Its extreme contagiousness and high mortality in certain classes of animals are comparable with the scourges of small pox in man and, in many other respects, the rabbit infection shows resemblances to this disease. In typical cases the characteristic features of small pox are duplicated and present the distinguishing signs of infection. The cutaneous eruption preceded by an erythematous rash and passing through the stages of papule, pustule and crust, the involvement of special organs, the complications and sequelae are similar in both diseases. The atypical forms of the two diseases also show similar modifications with lesions ranging from confluent eruptions to inconspicuous papules and systemic reactions varying from the complete absence of detectable symptoms to extreme prostration. The severity of the infection in pregnant and lactating females, the occurrence of abortions and the high mortality in young animals are especially significant.

Pathologically, the lesions in the skin of the rabbit were more deeply situated and resembled more closely those of the mucous membrane in man; they did not show the typical epithelial changes which have been described in the formation of vesicles and no typical inclusion bodies were found. In other respects the two diseases appear, both clinically and pathologically, to be essentially the same or identical. The absence of typical vesication and the failure to find inclusion bodies may be referable to the deep location and rapidly destructive nature of primary lesions. This assumption is borne out by subsequent experience with a less severe form of infection in which typical vesicles and inclusion bodies were present.

Characteristic lesions occurred with greatest frequency in the skin and mucous membranes of the mouth, nose and pharynx, in the lungs, liver, spleen, lymph nodes and bone marrow, and in the testicles and ovaries. In fatal cases of infection focal or diffuse lesions or both were nearly always present in these organs, but in some fulminating infections no lesions could be found in the skin.

Diffuse and focal lesions occurred together or as alternative processes, apparently depending upon the severity of the infection. In fulminating cases of disease, there was widespread degeneration and necrosis of cells comparable with those found in many infections and intoxications and in no way distinctive. The more common diffuse lesions, however, presented the characteristics of an acute inflammatory reaction in which extensive edema, a variable amount of hemorrhage, marked mononuclear but comparatively slight polymorphonuclear infiltration and widespread necrosis were distinctive features. Focal lesions consisted of clearly defined mononuclear infiltrations in which polymorphonuclear cells were almost entirely absent and edema and hemorrhage were less marked than in the diffuse form.

Focal lesions were intimately related to small blood vessels which usually showed varying degrees of endothelial damage. Diffuse lesions were also associated with vascular injury which, in cases not obscured by necrosis, was sometimes found widespread in small vessels and precapillaries. More frequently the damage was localized in single vessels, but was more intense than in focal lesions and accompanied by extensive edema and beginning necrosis in surrounding tissues, indicating that this type of diffuse lesion arose from extension of a focal process. In many fatal cases, however, necrosis was widespread and with loss of histological details, the original focal or diffuse nature of the lesion could not be determined. This was particularly true of pulmonary lesions which, in advanced cases, usually showed extensive consolidation with an exudate largely made up of mononuclear cells but also containing many polymorphonuclear cells and mass necrosis.

In view of the interest which has been manifested in the etiology of pneumonia in virus infections, it may be pointed out that lesions of this kind were not peculiar to the lungs. Similar processes were observed in the lesions of the skin which at times spread very rapidly with the development of a massive edema followed by necrosis and sloughing of the affected part. In these cases the exudate also contained a number of polymorphonuclear cells. Many of these animals survived, and it is worthy of note that the acute diffuse reaction was not followed by abscess formation as would be expected if the lesion were due to a secondary bacterial infection. Healing occurred as in the case of the typical focal lesion which supports the view that the diffuse reactions with edema, hemorrhage and infiltrations containing polymorphonuclear cells were produced by the action of the same agent which was concerned in the production of the focal lesions. From the information at hand, the action of a secondary invader cannot be excluded. At the same time, it must be remembered that the presence of a secondary invader does not prove that it played any essential part in the production of the lesion, and from the evidence available it appears that in this disease both the diffuse and the focal lesions were attributable to the same organism.

As a matter of fact, the diffuse reaction in the skin corresponds closely with the lesion usually obtained by a cutaneous or intracutaneous inoculation of vaccine virus, and any doubt concerning the etiology of pulmonary lesions is equally applicable to lesions of the skin, including those produced by inoculation with bacteria-free filtrates as well as the lesions seen in spontaneous infections.

Other lesions to which attention should be directed are those of the hematopoietic system, which in many fatal cases were so extensive that complete destruction of individual organs resulted. In such instances the spleen, bone marrow and many lymph nodes were so necrotic that microscopic identification depended rather on general outlines than on histological detail. The most constant finding, aside from the widespread necrosis in these organs, was the almost complete absence of polymorphonuclear cells. The sinuses of the spleen contained numerous mononuclear leucocytes but other forms were scanty, and in the bone marrow although their mononuclear antecedents were plentiful, polymorphonuclear cells themselves were extremely rare.

The remarkable healing of lesions of this disease in recovered animals is also worthy of mention. This was particularly noticeable in the organs of generation which, although most extensively affected, were repaired with restoration of spermatogenesis and ovulation. Cases were numerous in which clinical examination indicated universal and profound parenchymal damage. Yet such animals rapidly recovered normal functions and, in less than a month, were capable of active breeding service, and in general behavior could not be distinguished from normal animals.

Small pox was once regarded as among the most serious menaces to man, and in recognition of this fact protective measures were instituted which are unsurpassed by those employed against any other disease. Rabbit pox is clearly a serious menace to the rabbit and unless adequate precautions are taken, may seriously reduce the usefulness of the rabbit as an experimental animal. Typical cases of the disease are as easily detected as typical cases of small pox, but atypical and asymptomatic infections might escape detection or be mistaken for snuffles or for some innocuous disease. But because of their insidious and highly contagious nature they are a highly dangerous form of infection. Careful examination will, however, reveal sufficiently definite signs of infection in all forms of the disease to arouse suspicion or to make a positive diagnosis. From previous experience, it also appears that if sporadic cases of infection are detected before the disease has become epidemic, the infection can be suppressed by the destruction or isolation of infected and exposed animals. Such measures are apparently of no avail during the height of an epidemic.

The probable origin of the epidemic will be discussed in a subsequent paper. It may be said, however, that available evidence indicates that the infection which was responsible for the present epidemic was not introduced from the outside but originated in the Institute and was either masked by some other disease or spread in atypical form and thus escaped attention until it reached the breeding colony as an outspoken epidemic disease.

Finally, clinical and pathological evidence lead to the conclusion that rabbit pox is comparable with small pox in man and that it is produced by an agent closely related to the virus of small pox.

SUMMARY AND CONCLUSIONS

The lesions found in animals with epidemic rabbit pox have been described in this paper. The most distinctive gross lesion in all organs and tissues was the small nodule or papule which was found to consist of mononuclear infiltration and necrosis. Diffuse lesions were also found in which the infiltration was widespread and accompanied by edema, hemorrhage and extensive necrosis of affected tissues and organs. The possibility of the diffuse lesions being due to the action of secondary invaders was considered, but available evidence indicated that the different types, including pneumonia, represented reactions to a single causative agent. Moreover, an intimate relationship was observed to exist between lesions and small blood vessels in which primary endothelial damage was usually apparent. The degree of vascular damage generally corresponded to the extent of the lesion and it is probable that this in turn corresponded to the dose of the causative agent.

The close analogy between the clinical manifestations and pathological processes of this disease in the rabbit and small pox in man led to the conclusion that the disease in the rabbit is essentially the same as small pox, and that it is probably produced by a virus closely related to the virus of small pox. Available evidence indicated that the infection originated in the Institute and that it spread in atypical form or masked by some other disease until it reached the breeding colony as a clearly defined epidemic infection.

EXPLANATION OF PLATES

PLATE 22

FIG. 1. Undersurface of the skin from a fatal case of rabbit pox showing numerous papular lesions and a wide discolored zone of edematous infiltration. The variation in the size of individual lesions and the concentration of lesions in vascular areas as shown in this photograph were typical findings. Natural size.

FIG. 2. Undersurface of the skin from a fatal case of infection showing discrete lesions of the hemorrhagic type and their relationship to blood vessels. × 3/4.

FIG. 3. Hind quarters of a young animal killed late in the course of disease. Areas of hemorrhage and mononuclear infiltration were widespread throughout the skeletal musculature. Natural size.

PLATE 23

FIG. 4. Lungs from a fatal case of infection in a young animal showing numerous subpleural miliary nodules. Similar lesions were found deep in the substance of the lung but pneumonic consolidation was absent. Natural size.

FIG. 5. Liver and spleen from a young animal. Small focal areas of necrosis are distributed profusely throughout the liver. The spleen is enlarged and shows a few scattered discrete lesions. Microscopically, the sinuses were distended with large degenerating mononuclear cells and Malpighian corpuscles were poorly defined and consisted of similar elements interspersed with nuclear detritus. Natural size.

FIG. 6. Spleen from an adult animal showing marked enlargement and large subcapsular foci of necrosis. Lesions of this kind were unusual. Natural size.

FIG. 7. Lungs from an adult animal showing diffuse consolidation and large subpleural nodules with umbilicated centers. Compare with Figs. 8 and 9. Natural size.

FIGS. 8 and 9. Tongue. Large umbilicated nodules were commonly found in this situation. Microscopically, they consisted of subepithelial mononuclear infiltration which extended deeply into the underlying muscle, but the epidermis itself showed no specific changes. Natural size.

PLATE 24

FIG. 10. Section of the liver from an animal killed during the course of the disease showing degeneration of parenchymatous cells and an early focus of mononuclear cell infiltration in the portal connective tissue. In other sections all stages in the further development of this lesion could be traced from a more pronounced infiltration involving neighboring lobules to final necrosis. ×275.

FIG. 11. Section of bone marrow from a fatal case of infection in an adult

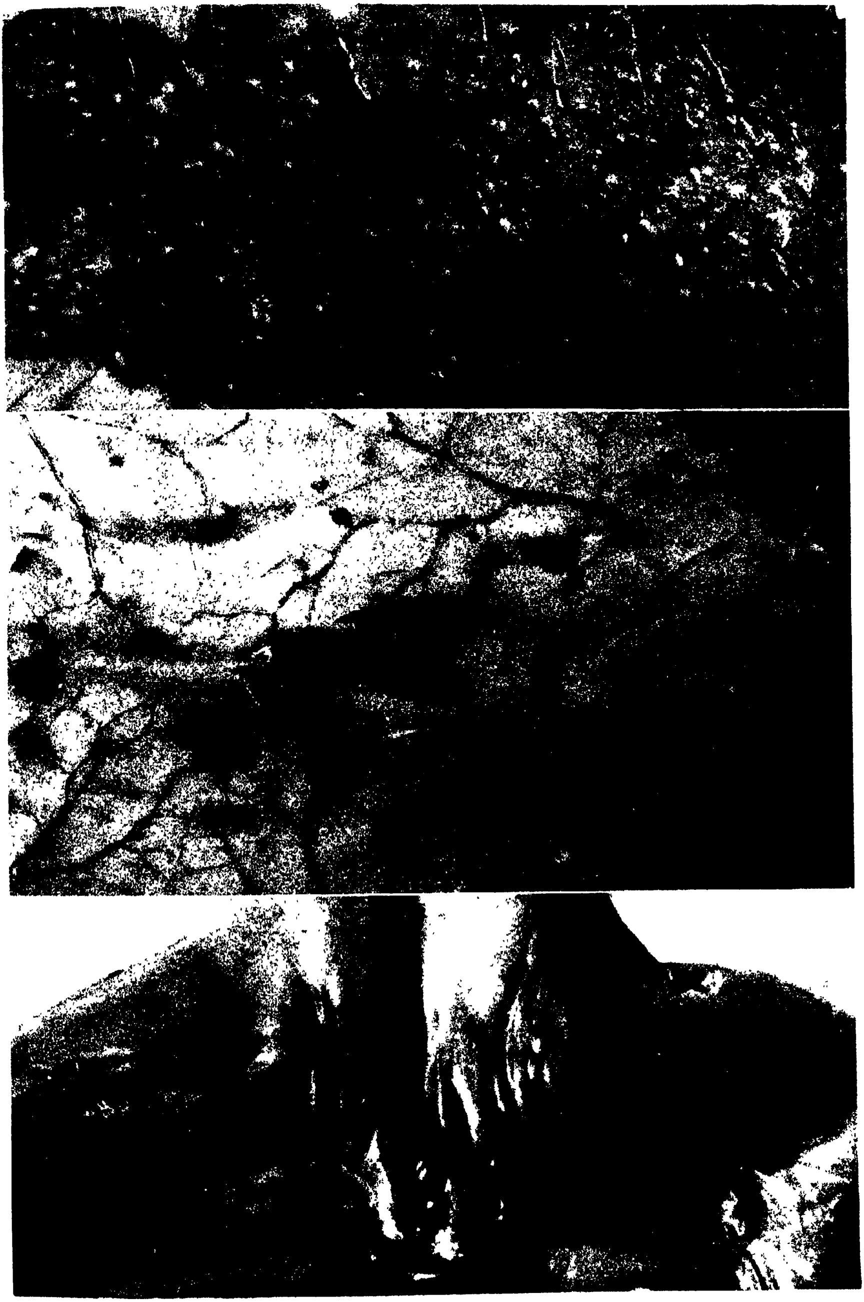
animal. There is widespread necrosis of hematopoietic tissue and no intact cells can be found. ×75.

FIG. 12. Section of lung from an animal killed in an early stage of disease. The lesion is deep in the substance of the organ and consists of a focal mononuclear infiltration with exudation into the alveoli and extension of the infiltration in alveolar walls toward the pleural surface. A blood vessel near the pleural surface contains an excessive number of mononuclear cells. ×90.

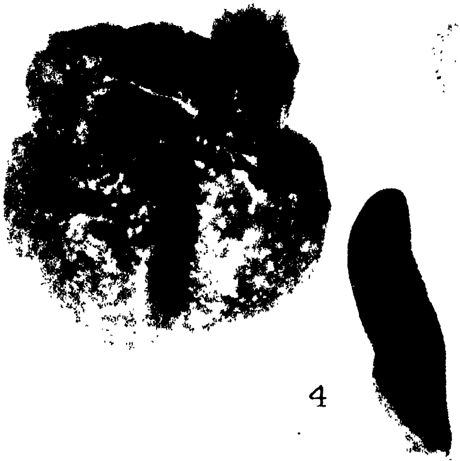
FIG. 13. Section of the lip showing a diffuse infiltration of deeper tissues including the muscle with mononuclear cells and fluid. ×130.

FIG. 14. Section through an early lesion in the skin of the ear showing mononuclear infiltration about a small blood vessel in the corium, destruction of endothelial cells and hyaline changes in the vessel wall. There were no specific epithelial changes. ×130.

FIG. 15. Section through a lesion in the lung of an animal killed when the first external sign of infection was observed. The lesion has progressed to necrosis but has remained localized about two adjacent blood vessels which show advanced changes in the endothelial and muscular walls. ×75.



(Greene: Rabbit pox. II)



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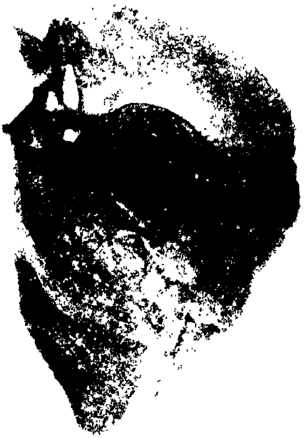

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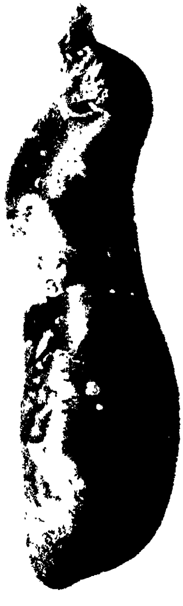

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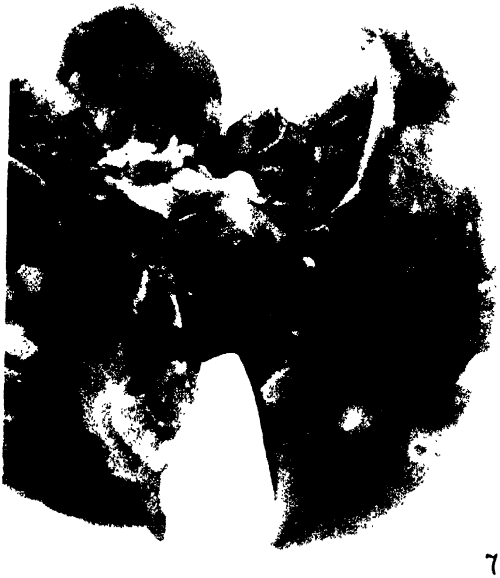

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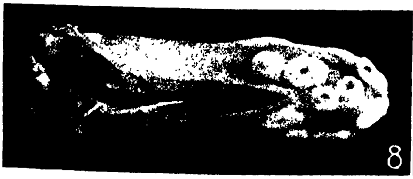

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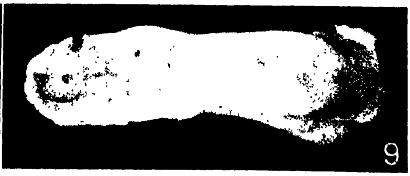

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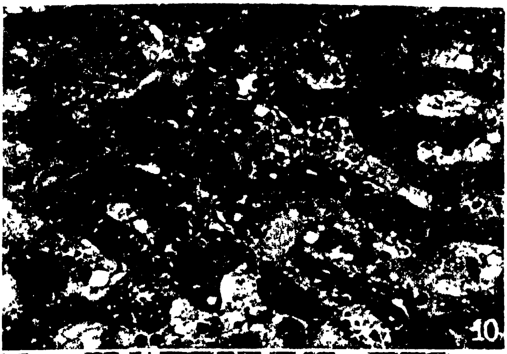

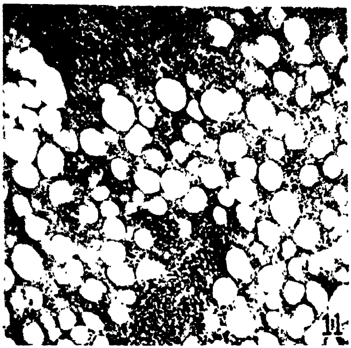

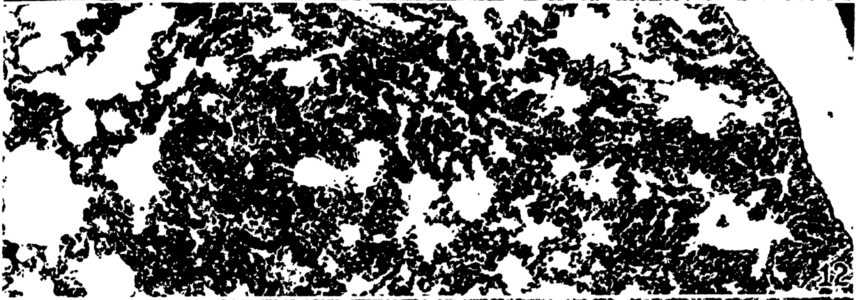

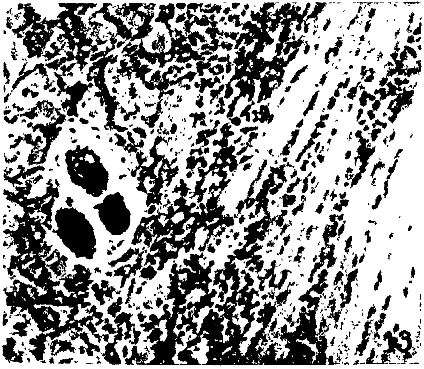

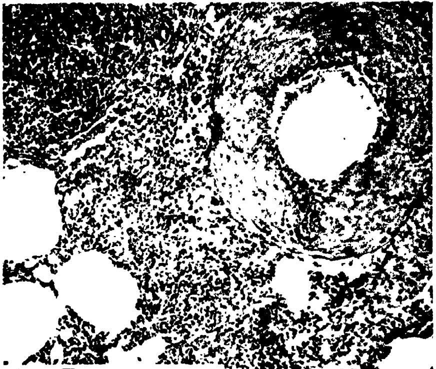

Photographed by Louis Schmidt

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ON THE EXISTENCE OF A FACTOR INCREASING TISSUE PERMEABILITY IN ORGANS OTHER THAN TESTICLE

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In an earlier publication a report was made on an infection-enhancing effect obtained with testicle extract. This property is shared by certain other organ extracts but to a far less degree (1). As the enhancing power of testicle extract was later found to be associated with a modification of the skin permeability (2), extracts from various organs have been tested to determine their relative ability to cause this.

The dissociation between *spreading* and *enhancing* action (3) observed with certain tumor extracts, and the necessity of ascertaining where the spreading factor exists in the body, led us to undertake a systematic study of extracts from various tissues by following the spread of India ink particles in the rabbit skin. Such an investigation was also considered as an indispensable preliminary to the study of the physiology of the factor or factors, and of the mechanism of its action.

Method

Adult rabbits of both sexes were used. The extracts were prepared by grinding the freshly removed organs with sand and 1, 1.5, and 2 volumes of Ringer's solution or water. The suspensions were centrifuged and 0.5 cc. of the cloudy supernatant fluid injected intradermally in the flanks of rabbits together with 0.25 cc. of Higgins' India ink, diluted 1:2. A similar control injection of 0.5 cc. of Ringer's solution or water plus 0.25 cc. of the ink was always made in the same animal. The spread of the ink was measured 1 and 24 hours after injection. In some cases precipitation of the tissue extract with 4 volumes of acetone, and re-extraction of the residue with variable amounts of water was resorted to with the hope of purifying and concentrating the factor. The technique, which had been very effective in the case of testicle extract, did not give better results than the use of fresh extract.

EXPERIMENTAL

About 120 tests were carried out, each test involving the extract of an entire organ, or in the case of some extracts, of the pooled organs of 16 different individuals (rats). The average results with testicle extract were included for comparison. The findings are summarized

TABLE I

Occurrence of the Spreading Factor in Various Mammalian Organs

Organ extract tested	No. of tests	Average spreading of 0.5 cc. organ extract plus 0.25 cc. of India ink dilution	Average spreading of 0.5 cc. saline plus 0.25 cc. India ink dilution (control)	Ratio of active spread to spread of control	Positive cases with increased diffusion
		sq. cm.	*sq. cm.*		*per cent*
Rabbit lung	7	27.3	7.2	3.8	100
" spleen	7	18.1	7.2	2.5	100
" liver	7	8.5	7.1	1.2	42
" kidney	10	17.6	7.2	2.4	80
" ovary	2	20.4	4.1	5.0	100
" placenta	2	22.0	9.0	2.4	100
" brain	10	10.4	5.3	1.9	90
" skin	4	10.1	6.5	1.5	100
" blood serum	15	9.8	10.9	0.9	0
" striated muscle	3	3.2	5.4	0.6	0
" testicle	9	55.6	5.8	9.6	100
Rat lung	2	20.3	7.8	2.6	100
" spleen	5	24.2	7.4	3.2	100
" liver	5	26.7	7.4	3.6	80
" kidney	5	18.6	7.4	2.5	100
" brain	4	14.5	6.6	2.2	100
" blood serum	2	7.8	7.8	1.0	0
" testicle	10	40.4	5.8	6.9	100
Guinea pig lung	2	17.0	6.1	2.7	100
" " spleen	2	11.0	5.0	2.2	100
" " liver	2	17.5	5.0	3.5	100
" " kidney	2	9.0	5.1	1.7	100
" " blood serum	1	6.8	6.8	1.0	0
Calf thymus	1	12.0	4.0	3.0	100
Human placenta	1	9.0	4.0	2.2	100
" serum	7	7.4	7.0	1.0	0

in Table I. They clearly demonstrate the existence of spreading factors in all organs studied, although in a proportion far inferior to that existing in testicle, as judged by the India ink spreads. Striated muscle extracts might constitute an exception to this rule. In one investigation they were found inactive.[1] Sera proved inactive in every case.

The quantitative differences in the spreading factor or the extracts studied were further brought out by dilution. Whereas testicle extract still increased the spread of ink when diluted to 1:400 (the highest dilution studied) extracts of other organs were no longer active at 1:10, and only in the case of lung and spleen extracts was there observed some spread at 1:5 dilution.

Another difference between testicle and other organ extracts consisted in the lack of regularity with which the latter yielded a spreading factor, contrasted with the perfect consistency of the former in this respect. This is indicated in the last column of Table I and holds especially for liver. Lung and spleen extracts, although fluctuating in their yields, always exhibited a certain activity in increasing the spread of ink.

The spread induced by the various organ extracts was similar to that obtained with testicle extract, but took place much more slowly. Nevertheless, lung and spleen extracts caused in certain cases considerable spreading in the hour following injection. In general though, the amount of the ink distributed through the area in which spread occurred was much less marked than in the case of testicle extract. The areas of spread when testicle extract was used looked always blackened by the ink, while the skin modified by the extracts from other organs, as shown by the spread of ink particles through it, was regularly lighter. Another detail to be mentioned is that the skin thickening (probably due to edema) was greater in the case of the latter. Skin treated with testicle extract was not swollen. Some of these points are illustrated in Table II, in which the areas and the density of the spreads produced by extracts prepared with the organs of a single rabbit were recorded 1 and 24 hours after injection.

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[1] Spinelli (6) has also found a spreading power in thyroid extracts. Muscle extracts, taken as control, were inert in 8 of his 15 trials. Testicle extracts were always active.

Summarizing we can state that practically every one of the 12 organs studied contains in widely varying amounts factors increasing tissue permeability, as shown by the spread through it of India ink, and that testicle and epididymis, lung and spleen seem to be the most active.

The possible rôle played by the testicle in bringing about the presence of the spreading factor in the other organs has been investigated. Their property of increasing tissue permeability was found to be retained after castration. The results obtained with rats' organs tested 5 weeks after castration have been included in the table. This observation indicates that the spreading factor is a usual constituent of these organs.

TABLE II

Progression of the Spread with Various Organ Extracts

	Readings after 1 hr.		Readings after 24 hrs.	
	Area of spread	Density of spread	Area of spread	Density of spread
	sq. cm.		*sq. cm.*	
Ringer's solution plus India ink (control)	4.1	±	4.2	±
Spleen extract	14.6	+++	29.0	+++
Lung "	9.0	+	36.9	+++
Kidney "	8.1	+++	17.6	+++
Brain "	6.2	++	10.5	++
Liver "	8.4	++	8.0	++
Muscle "	4.4	+	4.9	+
Testicle " (average of 9 tests)	19.4	++++	55.6	+++++

COMMENT

The results of the present investigation clearly demonstrate the existence in nearly every organ tested of a factor active in increasing tissue permeability. It was shown in previous work that the same organ extracts possess the power of enhancing bacterial and virus lesions (1). In the case of extracts from testicle (2) and from invasive bacteria (3) a direct correlation was found to exist between the degree of spreading and the intensity of enhancing power. These observations make it probable that the same correlation may exist in most of the organ extracts studied. Further experiments are necessary to test this assumption. It is sustained by the fact that certain azo compounds which are active spreading agents also enhance and increase the size of infectious lesions (4). The fact deserves mention, however, that spleen extracts not only failed to enhance vaccinal or staphylococcus lesions, but sometimes exhibited a definite inhibiting action. An analogous dissociation of the properties of organ extracts regarding the power to increase tissue permeability or to enhance infections has been met with in the case of certain melanomas (5). Likewise, extracts of some transplantable sarcomatous growths failed to enhance infectious lesions, although rich in spreading factor. In such cases there was an indication that an anti-infectious agent was present in the tissue extracts, in addition to the spreading factor.[2] For this reason we consider the permeability test more reliable for the detection of the factor in organ extracts than the original method of the production of lesions. Blood serum is completely devoid of both enhancing and spreading power.

Work is under way on the chemical relationship between the testicle factor and the factor obtained from other organs. The possibility of quantitative or qualitative variations at different periods in the life cycle is also being considered.

SUMMARY

Many of the organs from animals of both sexes, including the ovary, have been found to contain in various proportion a factor or factors increasing tissue permeability. The potency exhibited by such active extracts was always less than that of extracts from testicle. Blood serum was found to be devoid of any spreading property.

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[2] In support of this view we can adduce the fact that, while extracts prepared from testicle immune to vaccine virus still retain to the same extent as normal testicle the power of increasing dermal permeability, they fail to enhance the virus lesions and indeed actually suppress them. The immune testicle extract may have spread the vaccine suspension over a large area, but it has spread an inactivated material. It is worth noticing in this connection that Felton (7) has increased the virulence of pneumococcus by repeated automatic transfers in lung medium and decreased it by similar transfers in spleen medium.

BIBLIOGRAPHY

1. Duran-Reynals, F., *Compt. rend. Soc. biol.*, 1928, **99,** 6; *J. Exp. Med.*, 1929, **50,** 327. Duran-Reynals, F., and Suñer-Pi, J., *Compt. rend. Soc. biol.*, 1929, **99,** 1908. Pijoan, M., *J. Exp. Med.*, 1931, **53,** 37. Hoffman, D. C., *J. Exp. Med.*, 1931, **53,** 43.
2. Hoffman, D. C., and Duran-Reynals, F., *Science*, 1930, **72,** 508; *J. Exp. Med.*, 1931, **53,** 387. McClean, D., *J. Path. and Bact.*, 1930, **33,** 1045.
3. Duran-Reynals, F., *J. Exp. Med.*, 1933, **58,** 161.
4. Claude, A., *Science*, 1933, **78,** 151.
5. Duran-Reynals, F., and Stewart, F. W., *Am. J. Cancer,* 1931 **15,** 2790.
6. Spinelli, A., *Riv. patol. sper.*, 1932, **9,** 203.
7. Felton, L. D., *J. Exp. Med.*, 1932, **56,** 13.

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THE PARTICIPATION OF SKIN LYMPHATICS IN REPAIR OF THE LESIONS DUE TO INCISIONS AND BURNS

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Plates 28 to 30

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The lymphatics of human skin participate in the responses to very slight injury. As our previous work has shown (1), poorly diffusible, vital dyes, intradermally injected, enter the superficial lymphatic capillaries of the skin, and ordinarily are retained by them for several minutes before escape occurs into the interstitial tissue. Conspicuous increases in the permeability of the walls of these vessels, as manifested by an almost immediate escape of dye from the channels, follow such stimuli as the application of gentle heat, ultraviolet irradiation, or a firm stroke over the surface, too slight to abrade the skin. Studies of the lymphatic capillaries in the ear of the mouse have disclosed similar changes in the permeability of the walls of these vessels (2, 3). Here too a scratch insufficient to break the skin, a light stroke with a blunt instrument, exposure to ordinary sunlight, or an increase in temperature to but 5° above body heat suffices to induce a great transient increase in the permeability of the lymphatic wall.

The changes described have been effected without causing frank injury. In the present paper we wish to report upon the alterations in the permeability of the walls of lymphatic capillaries of the mouse ear in and about regions subjected to burns or incision. Our purpose has been to examine the responses of lymphatic capillaries under frankly pathological conditions, with special reference to the functioning of these channels during the formation and resolution of sterile inflammatory processes.

Methods

We have found the ear of the mouse an ideal structure in which to observe directly the changes in lymphatic permeability as they occur in living tissue.

As in the preceding work (3), mice of 16 to 18 gm. body weight were best suited to our purpose. In all of the experiments the animals' ears were observed at various intervals after injury had been produced under an anesthetic. When the interval was many hours or several days, ether anesthesia was used when the injury was produced and the final observations were made under complete sodium luminal narcosis. When the times of injury and final observation fell within a period of only a few hours, one anesthetization with sodium luminal sufficed for both. As in the earlier work, 0.125 cc. of a 2 per cent aqueous solution of freshly dissolved sodium luminal was given subcutaneously for each 10 gm. of body weight. All examinations were made under the binocular microscope by a method already described (2).

To render the lymphatic capillaries visible and to test for alterations in the permeability of the lymphatic wall, a highly indiffusible vital dye, pontamine sky blue (2, 3), was injected locally into the skin of the upper surface of the outer edge of the ear. It enters directly the lymphatics draining the region through rents caused by the injecting needle, and extends along the local network rendering it clearly visible (3). In all the experiments save where specific mention has been made, 0.1 cc. of fresh aqueous isotonic pontamine sky blue (21.6 per cent) was added to 2 cc. of a mixture of 1 part mouse serum and 3 parts Tyrode's solution, yielding a final mixture of approximately 1 per cent of dye in an isotonic menstruum of protein content similar to that reported for peripheral lymph (4–7). This was regularly used to obtain comparisons of the rates of dye escape from the lymphatics after different injuries or at varying intervals after a standard injury. It will be referred to as standard pontamine solution. When it entered the lymphatic channels of the normal ear, no perceptible dye escape took place from them for 10 to 15 minutes. In many of the present experiments, on the other hand, so rapid an escape of dye resulted in consequence of the injury to the lymphatics that we were led to employ a suspension of particulate matter to test whether or not there were openings in their walls. For this purpose dialyzed non-waterproof India ink (Higgins' American drawing ink), or else "Hydrokollag," was injected together with the dye solution. The method of preparation of these suspensions has already been described (2, 8). Further to compare changes in the permeability of the blood vessels of the injured ears with the changes occurring in the lymphatics, pontamine sky blue was injected intravenously into many of the mice. The dye was given as described in earlier papers (9–11), employing 0.05 cc. of a 21.6 per cent solution mixed with an equal volume of Tyrode's solution.

To study the physiology of the lymphatics during the progress and restoration of wounds or inflammatory processes, and to render our results comparable from animal to animal, it was necessary to devise methods of traumatization which could be standardized for repetition. This was accomplished in several ways to be described below. Heat was applied in such a manner that mild first, second, or third degree burns were obtained at will, the more severe burns resulting in necrosis.

The Lymphatic Changes Resulting from Skin Incisions

The effects of cutting through the skin were first observed. The incisions were made under the binocular microscope, so that the depth and extent of the wound could be accurately controlled.

The part played by the lymphatics in the process of wound healing is not known. In descriptions of the phenomena of wound healing there is little if any mention of these vessels. How do they behave during inflammation? What is their share in the healing of a wound? Do they become blocked like the blood capillaries when they are cut across? When they reconstitute during the healing process are the lymphatics more permeable than normal or less so? These and many other questions suggest themselves.

In our experiments the lymphatics of the skin of the mouse's ear were rendered visible by local injections of standard pontamine solution, at various times after incising the upper surface of the ear superficially or deeply. In the majority of instances healing took place rapidly and the behavior of the lymphatics could be studied during the healing processes.

Mice were anesthetized with ether and under the binocular microscope cuts about 1 cm. long were made half way between the tip and the base of one or both ears. In some cases the incisions were very superficial, involving only the corium and the subpapillary layer at most, and in these no hemorrhage developed. Probably only the most superficial lymphatics were severed. In other instances incisions were made deeply enough to cut the larger veins and arteries, while in some the upper surface was cut through to the cartilagenous plate of the ear. In still other experiments the cut went completely through the ear. Save in the animals with superficial incisions, more or less hemorrhage ensued, varying from small capillary ecchymoses to frank bleeding of the largest vessels.

A considerable number of mice were operated upon at one time, and at half hour intervals for 7 hours afterwards, groups of three animals each were anesthetized with luminal and local and intravenous injections of dye were made to render the blood vessels and lymphatics visible. On subsequent days other groups of six to ten animals were examined in the same way until all signs of wound reaction had disappeared.

The behavior of the lymphatics after incision differs strikingly from that of the blood vessels in certain respects. Where cut across the lymphatic channels remain open for a variable period of time, often for 24 to 48 hours or more, instead of constricting at once and closing as do the blood vessels.

Many of the blood corpuscles escaping into the tissue during the hemorrhage after incision pass directly into the lymphatics that are laid open, and continue to do so for many hours. Large numbers of erythrocytes are often found in the lymphatics both distal and proximal to the cut, as late as the day after making the incision. In instances showing this the lymphatics about the cut stand out clearly by reason of the contained blood. Clotting has not occurred and with a micro-spatula the intralymphatic cells can be moved in either direction. In relation to this finding, Clark and Clark (12) in their studies with transparent chambers in the rabbit's ear, report that holes made in lymphatics at the time of operation remain open several days.

When standard dye solution was injected at the ear margin beyond a transverse cut on the same day that the cut was made the channels at the periphery of the ear filled as usual but then emptied their stained contents directly into the incision where they had been cut across. Fig. 1 shows a photograph of the ear of a mouse injected in the usual manner 5 hours after a transverse incision. The wound became filled with blue dye which flowed without hindrance from the severed lymphatics. In a few experiments, instead of injecting the incised ears a tiny crystal of dye was pushed, under the guidance of the microscope, into a minute intradermal puncture wound at the periphery of the ear. The regional lymphatics took up the dissolving dye and within 15 to 20 minutes colored fluid could be seen passing from the severed lymphatic capillaries into the incision. Constriction and spasm of the small blood vessels severed were so immediate and effective, on the other hand, that often no bleeding occurred.

In eight instances within half an hour to 5 hours after a superficial cut had been made, a drop of standard dye solution was placed on the incision. In 3 or 4 minutes colored fluid appeared in the lymphatics on both sides of the wound. Those distal to it lying between the incision and the ear tip filled for the distance of a single pear-shaped segment only, that is to say to the nearest valve; those proximal, extending from the incision to the base of the ear, drained away dye solution toward the head. The finding was not invariable but was frequent enough to prove conclusively that the lymphatics were still open. Whether or not they took up dye seemed to depend upon whether it gained access to them through the fibrin clot in the wound.

The local injections at the ear margin showed not only that the severed lymphatics had failed to close but that the lymphatic capillaries distal to the cut but severed by it were more permeable than usual. Dye began to escape through the walls of these channels within 2 or 3 minutes after it had entered them, and always many localized dye ecchymoses were seen, as though the channels had received direct local injury, which was not the case (2). In the unharmed ear standard pontamine solution remains within the channels for 10 to 15 minutes before any visible escape takes place (3, 2).

In most instances in which local injections of dye were made at the outer ear margin after a transverse cut had been made, not only were those lymphatic channels filled which drained directly into the cut, but others which skirted it at either end. These intact vessels carried the dye as usual to the base of the ear but they also passed along some of it, by side branches, into the region directly proximal to the injury and even discharged some into the wound itself by collaterals connecting with the open lymphatics in its proximal margin. Like the channels distal to the incision, the proximal collaterals showed an increased permeability to standard pontamine solution, dye beginning to escape from them 2 or 3 minutes after it had entered. Both distally and proximally to the wound, therefore, the lymphatics were more permeable than elsewhere in the ear. Within 5 or 6 minutes after an injection the margins of the cut for a distance of 2 or 3 mm. were stained a bright, diffuse blue.

The results of intravenous injections of dye on the same day the cut was made furnished an enlightening contrast to these findings.

The animal gradually became blue all over save for an area of pallor in and about the incision for a distance of 2 to 3 mm. It was plain that occlusion of the severed blood vessels and spasm of those near-by prevented dye from escaping into the immediate region of injury. Just beyond the area of pallor, the blood vessels allowed more dye to escape than elsewhere in the ear, and within 5 minutes after an injection the pallid incision was surrounded by a ring of diffuse blue stain.

Sixteen animals were examined under sodium luminal anesthesia on the day following incision.

These had received superficial cuts in one ear,—extending only through the subpapillary layer of the corium,—and deep cuts in the other ear, reaching to the cartilaginous plate and severing the overlying blood vessels. In all instances both ears were hyperemic and moderately edematous, with the edema most marked distal to the incision, though definite on the proximal side as well. In such regions the ears showed "pitting upon pressure" by a blunt instrument. They were thick, the skin tense, and when punctured by a sharp needle fluid escaped. The wound was ringed by dilated, tortuous capillaries.

After injecting standard pontamine solution intradermally at the ear margin on the day following incision the lymphatics draining the region filled as usual and carried colored fluid to a point about 2 mm. from the incision. In most instances there was in this region a tendency for the lateral connecting channels to divert the dye to the extremities of the wound, and thence into main trunks leading around the incision toward the ear base. In four animals, though, dye solution also entered the wound showing that some of the severed lymphatic capillaries were still open. As a rule in each animal many dye ecchymoses occurred distal to the wound, but not elsewhere in the ear. Even 20 minutes after injecting the ear but very little dye escape had occurred from the lymphatics in the region proximal to the incision. In a few instances the channels distal to or beyond the cut and several millimeters from its edge were not only highly permeable while those proximal were not, but the former were often dilated and the latter constricted. In those animals in which dye solution from the lymphatics failed to stream directly into the cut, the slightest increase in the pressure of injection served to make it do so. The resistance to the flow of dye into the wound seemed to be located not at the edge of the wound itself, but 2 or 3 mm. away from it; for once dye had been forced through this region it readily entered the incision.

Fig. 2 shows the result of an injection of dye into the margin of an ear which had been incised 24 hours previously. The photograph was taken 5 minutes after the injection. Although some dye entered the incision, by far the greater part was shunted around it as described. The greatly increased permeability of the lymphatics distal to the cut is shown by the abundant escape of dye from these areas. As yet no dye escape had taken place from the channels proximal to the wound.

In four of the sixteen instances examined in this way, dye solution, injected at the ear margin, not only streamed into the wound but passed on directly through the incised region into the channels beyond. A photograph of the phenomenon is reproduced in Fig. 3. It shows the passage of dye in three channels either directly through the incised area or perhaps immediately under it and very close to it, with ecchymosis of colored substance into the incision. The extreme permeability of the channel walls distal to the incision and the lack of proximal dye escape are plainly to be seen.

Can it be that actual reconstitution of these channels had taken place or were they merely injured and not severed by the cut? The question is not to be answered from a single preparation such as the one shown.

To gain light upon it, the ears of six mice were incised transversely and the ear margin injected with dye. In each instance dye entered the incision through three or four channels but was not directly carried beyond, showing that the lymph channels had been severed. Diagrams were drawn to show the distribution of these channels, with the blood vessels as landmarks. The following day, when the ears were re-examined, the colored fluid in the distal portion of the charted lymphatics had completely disappeared. Dye injections were again made at the edge of the ear of three of the animals, inserting the needle through the same puncture wound and expelling dye in the same spot as before. In all, the same channels previously rendered visible by dye solution were again filled, and in addition, in each ear two or more channels unseen the day before. In two of the three instances one or two of the lymphatics which had filled on first injection and carried dye solution into the incision, now carried dye through this region into a continuation of the channel on the proximal side, allowing it to flow to the base of the ear. In the cut region profuse ecchymoses of dye solution took place immediately, and distal to the wound the lymphatics allowed dye to escape 3 or 4 minutes later, the normal interval between injection and dye escape being about 10 to 15 minutes. Proximally, no dye appeared outside of the lymphatics in 20 to 25 minutes.

Two of the remaining three animals were examined the next day and the last of them on the 3rd day after incision. In these similar results obtained save that with each day more channels were demonstrable traversing the wound area, and delivering dye solution to their continuations beyond. The evidence showed that the severed lymphatic capillaries reunited rapidly.

In four other mice bearing incisions 24 hours old intravenous injections of pontamine sky blue demonstrated an excessive permeability of the dilated tortuous capillaries and venules about the wound. Within 4 or 5 minutes the region was stained a deep blue by profuse dye escape from vessels there, but not elsewhere in the ear. No patent blood vessels entered the wound as yet. Nevertheless there appeared to be a rapid turnover of fluid within the incision; for, in the instances just described, when dye was brought into the incised region from the ear tip by way of the lymphatics, the resulting colored ecchymoses in the neighborhood of the injury disappeared far more rapidly than elsewhere in the ear.

In ten instances the usual routine dye test was postponed for 48 hours after making the wound. The cut ears were still edematous and hyperemic but the edema fluid was transparent.

In all these animals, after local intradermal injections of standard pontamine solution at the ear margin, dye failed to pass into the incised region. In six of the ten instances, no dye reached the margin of the wound, the lymphatic channels filling to a point 2 or 3 mm. distal to the cut and piping the dye through small lateral channels to other larger lymphatics which passed around it. When the pressure of the injection was increased slightly three or four lymphatic capillaries carried dye freely into the cut.

As in our earlier experiment, the resistance to flow of the dye toward the wound seemed located several millimeters distal to it and might be attributed either to pressure from the edema or to the existence of fibrinous plugs which were dislodged by the pressure. In four of the ten instances in which the pressure of injection was not increased some dye solution reached the incisions directly, colored fluid either pouring into the cut from the open ends of the channels or passing by way of a channel that was now intact, but, with profuse ecchymosis of dye into the cut. It was plain that in the wound area itself the severed lymphatic channels had either remained open or had reconstituted. They had not been blocked.

In all of the animals examined 2 days after making the skin incisions, the channels distal to the wound appeared much wider than those proximal, and the bulbous swellings were broader and larger. After entering the lymphatics dye began to escape within 4 or 5 minutes while from the proximal channels none appeared in half an hour.

The Minute Lymphatics in Later Stages of the Healing of Incisions

Daily, for 10 days, groups of four or five mice, taken from a number with ears incised at the same time, were anesthetized with luminal and examined for changes in the behavior of the lymphatics during the process of wound healing. The early findings differed but little from those already described, such differences as were noted being attributable to variations in the severity of the wounds rather than to the passage of time. A week after incising the skin of the ear edema was still present both distally and proximally to the wounds. The whole ear still showed moderate hyperemia. Under the binocular microscope many new-formed blood vessels were visible extending into the injured area, more of them on the proximal side. On both sides large numbers of tortuous blood vessels with rapid circulation were present, often with reversed venous flow in some of the larger veins.

The introduction of dye into the lymphatics disclosed a variety of changes. The channels distal to the cut were still far more permeable than normally, profuse dye escape through their walls beginning in 2 to 4 minutes after it had entered them. This was observed even 12 and 14 days after superficial cuts had been made in the ears. At this time the skin appeared healed to the unaided eye. The lymphatics appeared widely dilated either as a whole or in segments. The bulbs adjacent to the valves also appeared greatly elongated and widened, giving the appearance of aneurysmal swellings.

In all these experiments the region immediately next the healed edges of the incision was not entered by the dye, save in each instance by three or four channels which transported colored fluid through the incision into their continuations beyond. In passing the region of injury profuse ecchymoses of dye occurred, as in the dye experiments done 2 or 3 days after incising the ears. By far the greater amount was passed into lateral channels about 3 mm. distal to the incision, and by them to lymphatics which ran around the wound. Usually at the extreme ends of the incision where healing had just begun several trunks filled with dye and passed it on toward the base of the ear.

New Formation of Minute Lymphatics in Areas of Repair

Occasionally in animals tested on the 6th and 7th days, and characteristically in those examined later from the 8th day on, evidence of new formation of lymphatics was obtained.

When a local injection of standard pontamine solution at the margin of the ear was watched under the microscope one could nearly always observe the column of dye-stained lymph advancing down the lymphatics as usual. But a few seconds later, instead of stopping or being carried around the injury, it spread into a close network of very minute channels, lymphatic sprouts, forming a dense reticulum on the distal edge of the incision itself. Scarcely did dye reach these before it began to escape, long before any had come out from the channels in the uninjured portion of the ear. Within 1½ to 2 minutes the semicircle of the distal circumference of the incision assumed a diffuse blue. Such channels were never found in ears examined less than 6 days after an incision.

Fig. 4*a* shows an ear incised 9 days previously and now injected intradermally at the margin with India ink purified by dialysis. It was amputated at once after injection and photographed while submerged in neutral paraffin oil. At the left end of the incision, one sees several channels which conducted ink through the region of injury. On the right side reconstitution of channels, or a collateral circulation has not yet developed. In the incision itself many very narrow, linear, parallel lymphatics, situated in the new tissue filling the incision are disclosed by the ink. They lie at right angles to the main trunks, having the direction of the original cut. They are better shown in Fig. 4*b*, an enlargement of the same specimen. Unfortunately India ink, owing to its particulate character, fails to fill as many of the very minute lymphatics as a dye solution and it gives no true idea of the richness of the new-formed plexus. When standard pontamine solution was employed in such experiments it escaped so rapidly from the small vessels that good photographs could not be taken though the abundance of new-formed lymphatics was plainly disclosed.

When local injections of dye were made, 1/2 to 3/4 cm. *proximal* to the incision, similar networks of newly formed lymphatic capillaries were found in its proximal lip. Retrograde passage of dye occurred showing that the new capillaries were without valves. They were exceedingly permeable as shown by the almost immediate escape of dye. The old channels on the proximal side of the wound failed to allow dye to escape in half an hour.

To study the relation of the new-formed lymphatics to regenerating blood vessels, the ears of 20 mice were incised in the usual way and after 10 days the animals were anesthetized and injected intravenously with 0.05 cc. of isotonic aqueous pontamine sky blue solution. Two minutes after the injection was completed, a local injection of 2 per cent vital red in the usual menstruum of 1 part mouse serum and 3 parts Tyrode's solution was injected intradermally at the ear margin. The dense plexus of new-formed lymphatics about the wound filled with red dye and the newly formed blood vessels with blue. The two types of vessels entered the healing area about equally far and both were much more permeable than those elsewhere in the ear. In the center of the healing wounds neither new-formed blood vessels nor lymphatics were visible. In about half the instances the incisions were traversed by two or more larger lymphatics, which passed directly through or just beneath them. No blood vessels appeared in this area.

The Lymphatic Capillaries in and about Burned Regions

To study further the lymphatic capillaries in the periods of formation and recovery from inflammation, advantage was taken of the fact that sharply localized, standard burns of similar intensity could be made in the skin of the ear of the mouse.

Injury by Heat.—Anesthetics were used, as already described. To obtain comparable burns we adopted modifications of methods to apply mild heat stimuli to the ear of the mouse (2). Several thin-walled glass water chambers were blown in various shapes and sizes with a flat surface that could be placed against the animal's ear. Each possessed three openings, one for the insertion of a thermometer and two for circulation of water at the desired temperature. One of these chambers possessed a flat oval surface only 3 mm. in diameter. To produce localized burns, hot water was circulated through the chambers and the ears of the anesthetized mice were gently held against the flat surface. By varying the temperature of the water or the duration of contact of the chamber with the ear, first, second, or third degree burns could be produced at will.

"Stigmatic Burns."—Strictly localized, marked superficial burns, which we may term stigmatic burns, were obtained in another manner. An ordinary bac-

teriological platinum inoculating wire was heated at its mid portion by a microburner until the free tip glowed dull red. The etherized mouse lay with the ear resting on the tip of the index finger and the hot wire was quickly and lightly touched to the central part of the upper surface of the ear. Later at intervals the animals were reanesthetized with sodium luminal in groups of ten, as the experiments required.

Stigmatic burns usually healed rapidly in a few days but occasionally progressed to necrosis, resulting in a neat perforation of the ear, as though it had been punched out.

The burns produced by the small water chambers and the hot platinum wire involved but a small part of the animal's ear, at its middle.

Changes in the permeability of the blood vessels were determined as described earlier in the paper. To test lymph permeability, standard pontamine solution was employed in the usual manner.

The local injections of standard pontamine solution into the tissue at the margin of the ear resulted in its entrance into those lymphatic capillaries which lay in uninjured tissue but which passed through the burned regions and emerged again into normal tissue. Other channels not directly involved in the burn but surrounding it, both near and remote, also took up the dye solution. Control observations were made by injecting the uninjured ears of the same animals. As the processes of repair were often protracted, dye injections were made soon after injury in some instances, in others at half hourly intervals up to 6 hours,—and, finally, at daily intervals up to 10 days or 2 weeks. Always before injecting the standard pontamine solution, the condition of the circulation of the ear was determined under a binocular microscope. The mice lay in plastalene moulds with the ears lying horizontally on porcelain placques and illuminated as previously described (3, 9).

Experiments were done on four series of mice with the water chamber at 46.0°, 53.0–55.0°, 59.0–60.0°, and 67.0°C., respectively. In yet another large series of animals stigmatic burns were produced. It was found that increasing degrees of heat or a longer application simply increased the severity of the reaction. When the burns were severe enough to cause permanent arrest of blood flow the lesions progressed to necrosis within 36 to 48 hours. Such burns almost invariably followed application of the chamber to the ear for 1 minute with water circulating at 59.0–60.0°C., or for as short a period as 20 seconds with the water temperature at 67°C.

When a small spot of skin on the mouse's ear approximately 3 to 5 mm. in diameter was heated at 55°C. for approximately 1 minute moderately severe burns resulted. In the sharply localized region of injury circulation in the smaller vessels was stopped, and often, even in the largest radial veins and arteries, blood flow ceased temporarily. A few seconds afterwards a reactive hyperemia occurred about the heated area, which in 2 or 3 minutes extended over the whole ear. After a few minutes, pronounced edema formed in and about the burn, that is to say the ear became thicker there, the skin tense, and, to a blunt needle, there was "pitting on pressure." Puncture with a sharp needle led to the escape of fluid, and under the binocular microscope the edematous area assumed a ground glass appearance.

The Immediate Effects of Severe Burns.—When standard pontamine solution was injected into the outer margin of the ear, a few minutes after heating an area midway between the tip and the base, an extraordinary increase in the permeability of the walls of the lymphatics in the burned area was manifest. Dye passing into them from the normal channels nearer the margin at once escaped through their walls into the surrounding tissue.

Fig. 5*a* shows the escape of dye from the lymphatics in a burned area only 2 minutes after dye injection into an ear submitted to the water chamber at 55° for 40 seconds, 8 minutes previously. Such contact usually resulted in a third degree burn with temporary stoppage of the circulation for several hours and severe edema.

The region of dye escape from the channels coincides with the burned area. From the lymphatics in the uninjured portion of the ear there has been no escape of dye. In Fig. 5*b* the same preparation is shown 2 minutes later. Much more colored matter has now escaped from the channels in the burned area and it is distributed more widely.

In ten instances dialyzed India ink was injected together with standard pontamine solution into the margin of ears which had been burned in the same way. Although dye escape in the injured region was prompt and abundant the ink particles failed to pass out and the lymphatic capillaries were clearly defined by it.

Fig. 6 illustrates the immediate findings in a burn of lesser degree when there had been time for the formation of edema. The water chamber at 55°C. was in contact with the ear for 20 seconds. An hour later the standard pontamine solution was injected, the photograph being taken after another 4 minutes. The area of the burn, which corresponds with that of the escape of dye from the lymphatics, was already edematous, yet the channels penetrating it carried much of their contents into the uninjured tissue beyond, where no dye escape from the lymphatics occurred. Ink escaped from none of the lymphatic channels of such burned areas.

When the chamber containing water at 67°C. was applied to the ear of the mouse for 30 seconds or more, or when the ear was touched with the hot platinum wire, marked punctate burns resulted. As in the experiments in which moderate burns were induced, so in these, stasis in the injured area and reactive hyperemia round about occurred immediately. Edema followed.

When isotonic pontamine sky blue was injected intravenously 10 to 20 minutes after a pronounced contact or stigmatic burn, the blood vessels surrounding the ischemic burnt area were unusually permeable. Within 2 to 4 minutes after the injection, there could be seen about this area a collar of deeply stained tissue, colored by the escape of dye from the blood capillaries and venules close to the burn and long before escape from blood vessels elsewhere in the ear was visible. The tissue within the burnt region itself remained ischemic and therefore no dye entered it.

In contrast with these findings, following a local intradermal injection of dye at the margin of an ear burned in the same way, the lymphatics passed much of the colored fluid into the injured region itself where it escaped from the channels.

In twelve instances, at varying intervals from 3 minutes to 1½ or 2 hours after producing the burn, standard pontamine solution was injected into the skin of the ear, close to its outer margin. In more than half of these, lymph channels could be made out traversing the burned area, but the escape of dye from the portions of these within the burns was so immediate and so great that one might almost suppose that the cellular membrane of the lymphatic wall no longer existed as such. Yet, when the ear was reinjected, in five of these instances with dialyzed India ink or "Hydrokollag," the walls of the lymphatics were demonstrated to be still continuous, for none escaped. In three other experiments standard pontamine solution and India ink combined, injected 2 hours after the production of the burns, was seen to enter the lymphatics as usual; and these carried both dye and India ink through the punctuate, burned area into the lymphatics beyond, though there was some dye loss during the passage.

When the time interval between the production of the burn and the dye injection was increased to 4 or 6 hours, dye often failed to reach the ischemic injured area by way of the lymphatics. The dye was carried to within 2 or 3 mm. of the burn and there immediate and profuse ecchymosis of it occurred. The channels which passed around but close to the burn were extremely permeable so that it was surrounded by a dense cloud of diffuse blue color, while the edematous burn itself remained colorless. In two instances a lymphatic channel carrying dye did penetrate the burn and pass through. As result the patch was stained blue owing to the escape of dye from the lymphatic within it.

When dye escapes from lymphatic capillaries which lie outside an edematous burned area the diffuse blue color it gives to the tissue does not extend into the burn. When, however, dye is carried by a lymphatic into the edematous area, it escapes readily from the containing vessel. The edema fluid would seem to be partly made up of lymph which has escaped from lymphatic capillaries traversing the injured region.

Figs. 7*a* and 7*b* are photographs of an ear taken 3 and 8 minutes, respectively, after a marginal injection of dye. Three hours prior to this a third degree punctate burn was made (the water chamber for 45 seconds with water at 60°C.). In the first photograph, taken only 3 minutes after injecting the ear, the pale region of the burn had become edged with blue dye owing to escape from the lymphatics at its edge. A single large one carries dye directly through the burn itself and the dye escaping from it has merged with that from the ecchymoses on the right. The second photograph taken 5 minutes later illustrates the progress and extent of dye escape. After burns such as this the entire ear structure remains hyperemic for several days. Capillary dilatation in the region surrounding the burn is pronounced and the edema progresses until the ear may be half a centimeter thick. Under the microscope the blood vessels appear as if seen through ground glass.

Heating the skin to 67°C. for 15 seconds frequently gave rise to blisters. If dye was injected into the ear margin just beforehand, a very pronounced escape of dye took place from the lymph channels. The extravascular movement of dye under these circumstances was almost immediate, and it frequently extended several millimeters from the channels. The observation suggests that ordinary blister fluid is formed not only from vascular transudate but from a lymphatic one as well.

The findings as a whole furnish evidence for the participation of the lymphatics in the formation of and recovery from edema, confirming the observations of an earlier paper (2). Further work on this theme is now in progress.

The Lymphatics in the Early Stages of Restoration and Repair of Superficial Burns

Under ether anesthesia, stigmatic or contact third degree burns were made on one ear of a large number of etherized mice. Each day thereafter groups of eight or ten animals were anesthetized with sodium luminal and a dye injection made intravenously or into the ear margin. As controls the normal ears of the same animals were used.

The findings during recovery from both types of burns were so similar that no attempt will be made to distinguish between them.

Twenty-four hours after the formation of stigmatic burns, each was surrounded by an area of pronounced hyperemia and edema. Under the binocular microscope the whole ear showed a mild reactive hyperemia with dilatation of capillaries and venules about the burn and severe edema at the margin of the latter. Stasis in the blood vessels round about varied in degree, but in all instances the region of actual injury was ischemic.

In these instances and in experiments to be detailed there were many findings similar to those occurring after incision of the ear. After a burn, as after incision, there was a strong tendency for dye injected at the ear margin to be carried around the region of injury by numerous enlarged lymphatic channels. During the first 24 hours after the injury these, when they contained dye, showed a greatly increased permeability, with profuse ecchymotic dye escape wherever a lymphatic approached the injured region itself.

Occasionally, lymphatics transported colored fluid directly into and through the burned areas as through incisions. Though the areas were edematous, dye escaped so rapidly into them that one might doubt the existence of lymphatic walls, were it not that India ink did not escape. However, in most cases the channels surrounding the burn led most of the dye around it. Those immediately next the burns seemed to terminate in blind ends which may have been closed, perhaps by heat coagulation or thrombosis. Only when the injections were made with some pressure was colored fluid forced into the burn itself. Then, if such a burn had been made 2 to 6 days previously, the lymphatics failed to carry dye completely through it into the proximal normal tissue, but instead an ecchymosis of dye resulted within the injured area itself.

A further similarity in the reaction of lymphatics after injury by burns and incision was observed in the fact that 24 to 30 hours after burning the ear the lymphatics proximal to the burn appeared as sharply outlined bands showing no escape of dye into the surrounding edematous tissue, even 20 to 25 minutes after an injection. The color within the channels became rapidly paler as though the contents had been diluted and cleared by an intake of fluid. Instead of the excessive escape of dye from the lymphatics within the burn and distal to it, there was an apparent failure of dye escape in the proximal tissue.

The Lymphatics in the Later Stages of the Healing of Burns

Two days after a burn had been made, the lymphatics which filled most readily, that is to say those which skirted the burn, lay within the hyperemic area surrounding the injury. Little or no apparent escape of dye occurred from them, although they were usually somewhat dilated. The contents were more rapidly carried away than in normal channels, to judge by the fading of the blue color within them, and without visible dye escape. The few ecchymoses which did occur in the region disappeared in 10 to 15 minutes, but distal to the injury they often persisted for several hours. The observation afforded further evidence of an increased lymphatic turnover going hand in hand with hyperemia and increased vascular permeability.

In five instances this last was easily demonstrated by intravenous injections of dye solution. Dye poured from the dilated capillaries and venules about the burn long before it escaped in quantity elsewhere in the ear. As result a bright blue ring about 4 mm. thick was formed surrounding the colorless area of injury. Fig. 8 shows the result of such an experiment, photographed 4 minutes after the intravenous injection was made.

On the 4th day, local and intravenous injections of dye into such animals yielded findings similar to those just reported.

The blood vessels seemed still to be more permeable than normal, judging by the rapidity of dye escape, but one could not say whether this was due to a more permeable state of the vessels or to an increased supply of stained blood. The lymphatics too were more permeable than normal in the region distal to the burn, and here they seemed wider than those proximal to it. When dyes entered the channels completely surrounding the burned area, profuse escape occurred distally, forming a blue cresent of diffuse staining. The proximal lymph vessels seemed less permeable than normal, for within half an hour after their injection the blue-stained contents had become pale, without showing any visible escape of dye. Whether the failure of dye escape is due to decreased permeability of their walls or to brisk fluid movement into the vessels from without cannot be said. In either case the phenomenon shows drainage of the tissues by the lymphatic capillaries.

The findings in no way differed 5 days after producing the burns but by the 6th and 7th day the inflammation seemed to have subsided. No longer was there evidence of hyperemia in the ears, save for a few dilated capillaries immediately about the injured area. The edema had resorbed, leaving only a small swollen region in and about the burn, which by this time had become indurated. Nevertheless the usual local injections of pontamine solution showed the lymphatics to be still excessively permeable. Between the burned area and the ear margin numerous local ecchymoses of dye occurred, as profusely and rapidly as on the 1st and 2nd day after injury.

New Formation of Lymphatics within Burns

By the 7th or 8th day of healing evidence was obtained of new formation of lymphatic capillaries, as in our experiments with incisions.

When dye injected at the ear margins reached the burned areas, some of it entered a dense, ramifying, twig-like plexus of lymphatic capillaries within the distal edge of the burn itself. Some dye passed through in large channels, the remainder was carried around the injury. Fig. 9 shows such a new-formed network about a burn 9 days old. The photograph was taken 2 minutes after injection of the ear with a suspension of India ink. Only a few of the fine, hair-like projections extending into the new-formed tissue are visible; for ink suspensions did not yield a complete injection of these vessels.

To show the relationship of the new lymphatic capillaries to blood vessels intravenous injections of pontamine blue solution were followed in 1 or 2 minutes by injections of vital red in the ear tissue, as in our earlier work with incisions. The findings were so much alike no separate description will be given.

Similar experiments were made during the later stages of healing. Lymph channels were found completely traversing the burns. The sequence of events when dye was introduced into them has already been described.

DISCUSSION

In our previous papers attention has been called to alterations in the permeability of the lymphatic walls occurring under the conditions of the everyday life of the animal. Even very mild thermal and chemical stimuli result in the escape of substances of large molecule, for example hemoglobin, which are retained within the lymphatics of an unharmed ear. On the other hand true particulate matter, for example India ink, fails to escape, showing that no lacunae exist in the lymphatic wall. In the present paper we have reported upon the changed conditions in the lymphatics following incision and frank injury of other sorts. Profound alterations in the permeability of lymphatic walls, in and about these injured regions, speak for an active participation of the lymph system in the changed processes of fluid exchange. What can be inferred concerning the rôle of the lymphatics during inflammation and repair?

Our experiments make plain the fact that, like the blood vessels, the lymphatics respond to injury first by pouring their contents into the region involved. During the first few minutes or even hours after an injury the permeability of the lymphatic wall is enormously increased without loss of its anatomical continuity, at least under the conditions of the experiments described. The most indiffusible vital dyes pass out readily but ink particles are retained. The lymphatics are so permeable that it is difficult to suppose that they can still function as channels for the conveyance of fluid. That they fail to do so adequately is indicated by the developing edema. The lymphatics are rendered more permeable for a considerable distance around the immediate region of injury. Inflammatory edema is obviously due to a change in the lymph vessels as well as in the blood vessels. The nature of the various changes is not understood. Soon after a burn the lymphatic capillaries distal to the wound appear dilated and those proximal either constricted or normal,—the former more permeable than the latter. The increased permeability may follow dilatation. Yet obvious changes in permeability have been observed which appear to be functional and not anatomical. Thus, the lymphatic capillaries, skirting a burn or wound and not directly affected by it, neither dilated nor constricted, are usually for the first 48 hours much more permeable than normal.

Our work indicates that the brief isolation of a skin region following injury, an isolation resulting from the excessive permeability of both lymphatics and blood vessels, is first lessened when the lymphatics begin to function again. Their severed ends, unlike those of the blood vessels, remain open and lead away fluids from the wound. The fact that lymphatics remain open and the blood vessels closed in an incision goes far to explain the frequent infection by way of the lymphatics. Soon after an injury in the skin of the ear, the lymphatics lying between the head and the injured region regain their normal permeability and apparently function as draining and conducting channels. Through these and not through the blood vessels resorption from the wound first begins. Through the lymphatics toxins or noxious products of disintegration are first carried away to be sieved or passed through the lymph glands before reaching the body at large. The evidence for this fact, though by no means complete, is sufficient.

In this connection, it should be recalled that we have been dealing with a type of lymph drainage slightly different from that in human skin. The ear of the mouse is very thin and both the superficial and deep plexuses of lymphatics lie close to the surface. As result the lymphatics usually injected lie in the deeper plexus. It is possible that the current in these channels is more rapid than in the superficial lymphatics of the skin of man, that is to say, the lymphatic capillaries which become filled by intracutaneous injections of dye (1). There results in the mouse ear a lymph current just beneath the epidermis which probably does not exist in the human skin, for there the intradermally injected dye passes, after running 2 or 3 cm. in the superficial channels, into a deeper layer (1). However this may be, the fact should be stressed that the lymphatics injected in these experiments with mice probably correspond in function with the deeper plexus found in the subcutaneous tissues of man, lymphatics which are sufficiently superficial to be involved by any cut causing hemorrhage, or by a third degree burn. In these deeper lymphatics in man there is, of course, a considerable flow (1).

The walls of the lymphatics directly draining a burned or incised area, after having at first been abnormally permeable, within 48 hours after the injury became far less permeable than normally for diffusible substances. At this time intravenous injection of dye shows the blood vessels in the injured region to be still abnormally permeable and, owing to hydrostatic conditions, they must still be adding to the accumulation of tissue fluid. The tissue is edematous and rapid continuous passage of fluid must be taking place through the lymphatics, for dye solution placed within them quickly pales and is swept away. In the later stages of healing, 48 to 72 hours after an ear injury, there is a definite tendency for lymph peripheral to the injured spot to flow around it, instead of through it. Dye injected into the distal channels is carried to a point close to the injury and then is shunted around it. It is as though the lymphatics were obstructed by wound materials or perhaps plugged, as has been suggested by Menkin (13) in his work upon blood vessel changes in large areas of inflammation. Evidence of a participation of the lymphatics in repair is seen in their tendency to rapid reconstitution and a new formation of them not only in the regions where they have merely been severed but where they have been injured or destroyed by burns.

Great numbers of new lymphatics were seen in the regions recovering from burns. When the latter had been severe enough to cause necrosis of the ear and perforation many new tiny lymph capillaries were found in the granulation tissue of the healing margins obviously taking their place in actively growing tissue. But in the tissue far removed from the healing margins, though still within the burned or incised regions, as shown in Figs. 4*a* and *b* and Fig. 9, many more lymphatic channels became visible after dye injection than in similar areas of normal ear. As the photographs show, some were large and some small. In an earlier paper (3) we have shown that the superficial lymphatic plexus is only partially filled by dye injections, that just as Krogh has shown for blood capillaries, small lymphatics temporarily closed and invisible may be teased open to allow dye to enter. As result one cannot say whether the excessive number of dye-filled lymphatic capillaries in the burned area signified a new formation of channels or a flooding of the entire lymphatic plexus, usually but partially visible after intradermal injection of dye and now entirely filled in a tissue engaged in active fluid exchange. When such burns were examined several days later, the number of dye-containing lymphatics had decreased to the normal number. Whether the channels were cut off or had closed down in a relative period of rest, cannot be said.

The observation that new collateral lymphatics can form is of course not new. It occurs after ligature of the thoracic duct (14, 15) and of large lymphatics of the limbs (16). Regeneration of lymphatics in the ear of the rabbit has also been demonstrated (17) as further their growth into granulation tissue (18). Union of lymphatics has been described by E. R. Clark (19) in the tadpole's tail and by Clark and Clark in transparent chambers in the rabbit's ear (20, 12).

In the past the speed with which a new formation of lymphatics occurs has not been recognized nor has it been appreciated that lymphatic drainage may be instituted from areas of repair before drainage takes place by way of the blood vessels.

SUMMARY

With the aid of solutions of vital dyes the lymphatic capillaries in the ear of the mouse have been studied during the period of immediate reaction to injuries of various sorts and during the period of repair.

The behavior of lymphatics severed by incision differs greatly from that of the small blood vessels. Instead of closing they sometimes remain open for as long as 48 hours. Materials introduced into the wound pass directly into the lymphatics through their gaping ends, a fact which will explain why infection from incisions is predominantly along the lymphatics.

All around an injury the lymphatics are rendered abnormally permeable. So, too, are the blood vessels, a fact well recognized in the past. Twenty-four to 48 hours later, at a time when the blood vessels in the edematous tissue surrounding the injured area are still much more permeable than normal, the draining lymphatics allow far less to escape than usual. The possible reasons for this have been discussed. The lymphatics participate in the removal of fluid from the edematous tissue.

As repair after injury takes place severed lymphatics may reunite when as yet there are no functioning blood vessels. Later an active hyperplasia of the lymphatic channels occurs, an extraordinarily abundant plexus of minute lymph capillaries budding into the area under repair.

BIBLIOGRAPHY

1. Hudack, S. S., and McMaster, P. D., *J. Exp. Med.*, 1933, **57**, 751.
2. McMaster, P. D., and Hudack, S. S., *J. Exp. Med.*, 1932, **56**, 239.
3. Hudack, S. S., and McMaster, P. D., *J. Exp. Med.*, 1932, **56**, 223.
4. Starling, E. H., *J. Physiol.*, 1895–96, **19**, 312.
5. Drinker, C. K., and Field, M. E., *Am. J. Physiol.*, 1931, **97**, 32.
6. Field, M. E., and Drinker, C. K., *Am. J. Physiol.*, 1931, **98**, 378.
7. Drinker, C. K., and Field, M. E., Lymphatics, lymph and tissue fluid, Williams & Wilkins Co., Baltimore, 1933.
8. Higgins, G. M., and Murphy, G. T., *Anat. Rec.*, 1928, **40**, 15.
9. McMaster, P. D., and Hudack, S. S., *J. Exp. Med.*, 1932, **55**, 417.
10. Hudack, S. S., and McMaster, P. D., *J. Exp. Med.*, 1932, **55**, 431.
11. McMaster, P. D., Hudack, S. S., and Rous, P., *J. Exp. Med.*, 1932, **55**, 203.
12. Clark, E. R., and Clark, E. L., *Am. J. Anat.*, 1933, **52**, 273.
13. Menkin, V., *Arch. Path.*, 1931, **48**, 249.
14. Lee, F. C., *Bull. Johns Hopkins Hosp.*, 1922, **33**, 21.
15. Colin, G., Traité de physiologie comparée des animaux, Paris, 2nd edition, 1873, **2**, 238, cited by Lee (14).
16. Reichert, F. L., *Arch. Surg.*, 1926, **14**, 871.
17. Eloesser, L., *J. Am. Med. Assn.*, 1923, **81**, 1867.
18. Coffin, T. H., *Bull. Johns Hopkins Hosp.*, 1906, **17**, 277.
19. Clark, E. R., *Anat. Rec.*, 1922, **24**, 181.
20. Clark, E. R., and Clark, E. L., *Am. J. Anat.*, 1932, **51**, 49.

EXPLANATION OF PLATES

PLATE 28

FIG. 1. Photograph of the ear of a living anesthetized mouse, injected at the ear margin with standard pontamine solution, 5 hours after a transverse incision had been made in the skin of the upper surface. The dye entered the lymphatics of the injected area and gradually extended along them to escape from their severed ends, filling the wound with blue dye. ×6.

FIG. 2. The result of an intradermal injection of standard pontamine solution into the margin of a mouse's ear, which had been incised 24 hours prior to the injection and photographed 5 minutes after it. The blood vessels and lymphatics had been cut through. Some of the dye reached and entered the incision but most of it was shunted around it as described in the text. The lymphatics are markedly permeable distal to the incision, as indicated by the abundant dye escape there, and much less so proximally. ×10.

FIG. 3. Lymphatics in the incised ear of a living mouse photographed 5 minutes after an injection of standard pontamine solution. The incision was made the day before and is easily seen in the photograph. Three lymphatic channels have conducted colored fluid past the incision into the tissue at the base of the ear. In doing so much dye has escaped, either into it or just beneath it. The increased dye escape distal to the incision, and the lack of escape proximal thereto is also well shown. ×10.

FIG. 4*a*. Demonstration with India ink of the lymphatic plexus about a healing wound. Nine days previously the skin had been incised; healing was progressing well. Immediately after a marginal injection of ink the ear was amputated and photographed under neutral paraffin oil. On the left side of the incision several lymphatics are seen carrying ink either directly through the healing incision or just beneath it. In the injured area a few very fine lymphatics can be made out. Linear and parallel, they lie transversely in the healing incision itself. ×10.

Fig. 4*b*. An enlargement of the same specimen. In this figure these newly formed lymphatics are seen to better advantage. ×40.

PLATE 29

FIG. 5*a*. This photograph shows the escape of pontamine solution into a burned region. Eight minutes prior to taking the photograph a chamber containing water at a temperature of 55°C. was brought into contact, for 40 seconds, with an area midway between the tip and base of the ear. Six minutes later the dye injection was made near the tip and 2 minutes later the photograph was taken. The area of dark, fuzzy escape of dye along the lymph channels coincides with the area of the burn. Elsewhere no dye has passed out. ×9.

FIG. 5*b*. The same preparation 2 minutes later, that is to say 4 minutes after the injection. In the unharmed ear standard pontamine solution does not begin to escape from the lymph channels for 10 to 15 minutes. ×9.

FIG. 6. Ear burned in a spot midway between the tip and the base by 20 seconds contact with the water chamber containing water circulating at 55.0°C. One hour later standard pontamine solution was injected at the tip of the ear and 4 minutes afterwards the photograph was taken. The lymphatics, though allowing some escape of dye into the burned region, where it colors the edema fluid, have transported much of it into the proximal, normal tissue. ×20.

PLATE 30

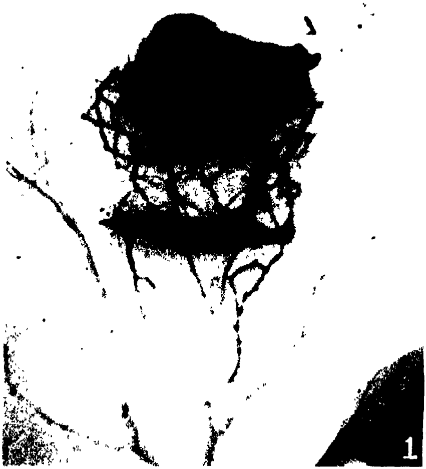
FIGS. *7a* and *7b*. Two photographs of the same preparation taken 3 and 8 minutes respectively after a local injection of pontamine solution at the margin of the ear. Three hours prior thereto a severe third degree punctate burn had been made midway between the tip and the base of the ear as described in the text.

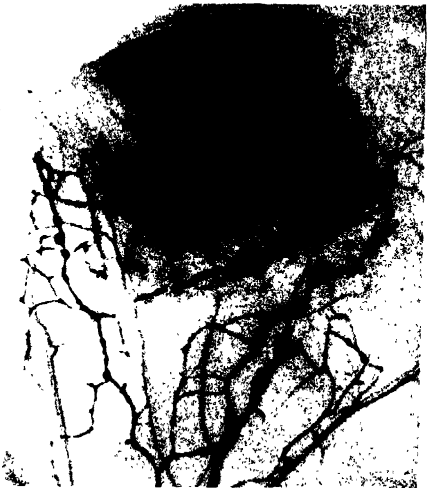
The rapid dye escape from the lymphatics close to the burned region is shown and the fact that the latter is almost unstained. A large channel traversed it from which dye ecchymosis occurred after a time. ×6.

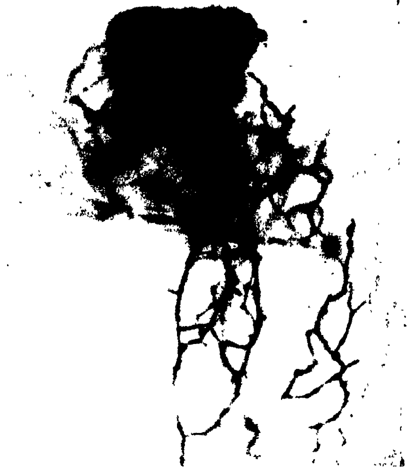
FIG. 8. A photograph of the ear of a living anesthetized mouse 2 days after a stigmatic burn had been induced on its upper surface as described in the text. Four minutes prior to the photographic exposure the animal received intravenously 0.05 cc. of 21.6 per cent aqueous isotonic pontamine sky blue solution.

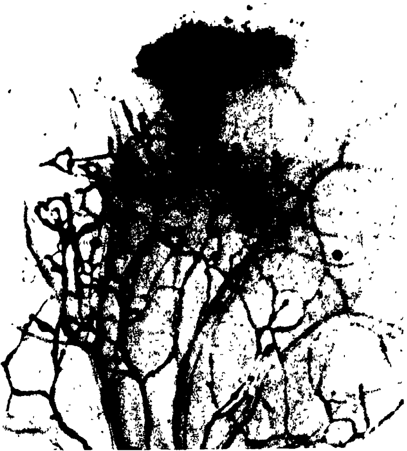
The increased permeability of the smaller blood vessels is evidenced by a ring of intense color about the burn, while elsewhere in the ear very little dye has escaped. At the center of the burned area there is some slight diffuse staining. ×6.

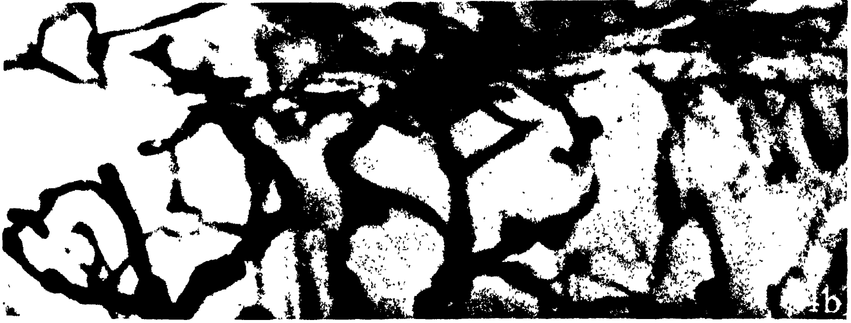
FIG. 9. The ear of an anesthetized mouse injected with a suspension of dialyzed India ink in 5 per cent gelatin solution 9 days after a stigmatic burn. One minute after the injection the ear was severed, placed under neutral paraffin oil, and photographed after another minute. The burn had caused a complete perforation of the ear which at the time of the injection was gradually being closed by granulation tissue. Several very small new-formed lymphatics can be seen in the new-formed tissue and about the healing burn there is an abnormally rich plexus of lymphatics many of which are very small. ×6.



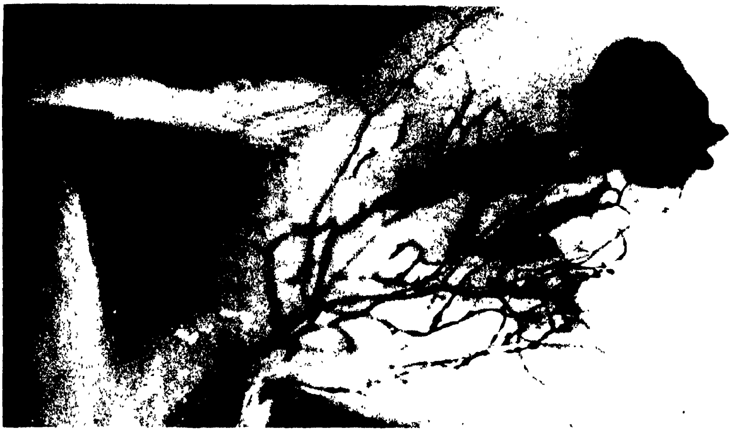








(McMaster and Hudack: Skin lymphatics in repair of lesions)



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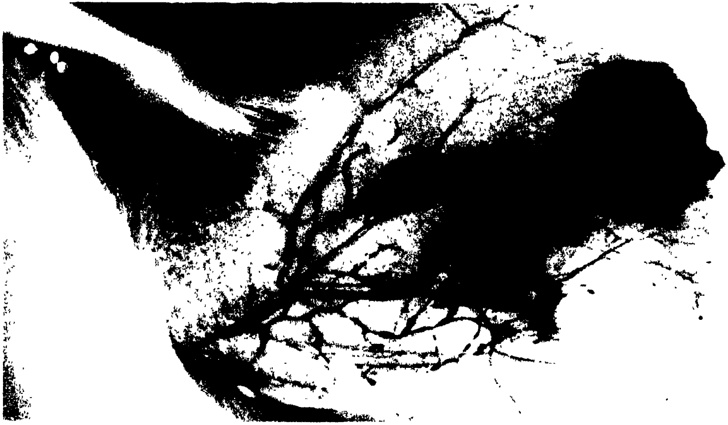

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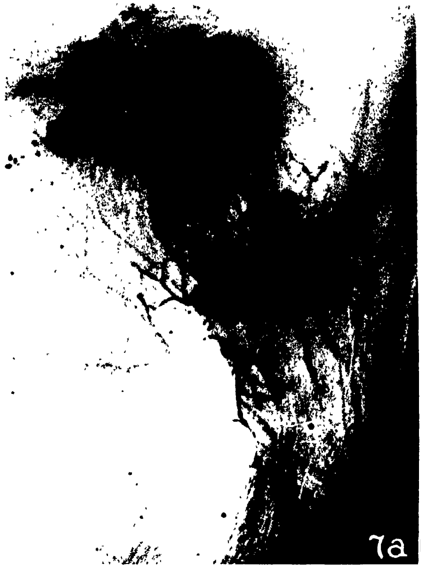
(McMaster and Hudack: Skin lymphatics in repair of lesions)



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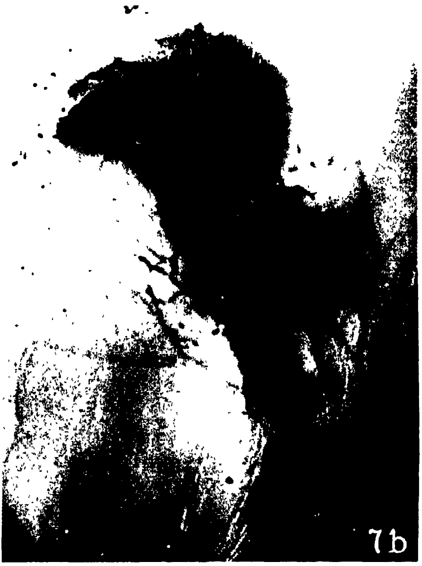

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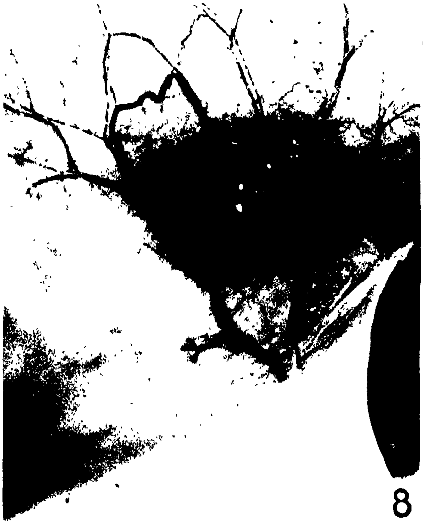

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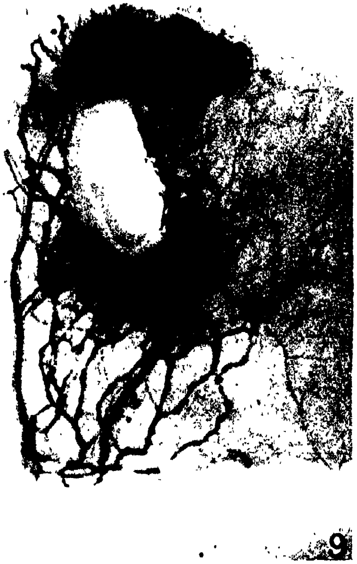

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IRREVERSIBLE CHARACTER OF THE LATE CHANGES AFTER HEPATECTOMY

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Death invariably follows extirpation of the liver in mammals. The first symptoms which arise are due to hypoglycemia, and vanish upon administration of glucose (1–5). For several hours, if sugar is given, the animals show no symptoms but later, despite all treatment, a second clinical stage develops, characterized by ataxia, weakness, coma and respiratory failure and ending in death (4, 5). The sequence of events is the same in the rabbit as in the dog (6–8). As the cause of the fatal issue is unknown, the work to be reported here was undertaken in an attempt to gain insight into it. We have endeavored to learn whether rabbits manifesting the symptoms of the advanced or "second stage" of liver insufficiency can be aided by the circulation of their blood through the livers of healthy animals or by cross-transfusion with normal animals. A preliminary note based upon some of this work has already been reported elsewhere (9).

Results of Circulating the Blood of Liverless Rabbits through a Normal Liver

Control Experiments.—Before attempting experiments with hepatectomized animals, trials were made with etherized normal ones. A carotid artery and external jugular vein were connected with the portal vein and hepatic vein respectively of the freshly removed livers of other rabbits and the blood was allowed to run through the organ for periods ranging from 2 to 6 hours. The technique will be described below. During the tests flow was free through the livers and the current in the intralobular capillaries could be observed with a microscope. The large amounts of bile secreted by the transfused organs gave evidence of their functional integrity. Sections of the hepatic tissue taken at the end of the experiments showed no changes from the normal appearance; and the healthy rabbits which furnished blood showed no ill effects whatever.

We next endeavored to determine whether the passage of liverless animals' blood through the liver of a normal rabbit would bring about clinical improvement after the symptoms of advanced liver insufficiency had developed. In all, fourteen experiments were made, of which four were carried to completion without any perceptible faults of technique which might render the findings questionable. The results of the other ten experiments were confirmatory of these four.

Method

Male rabbits 1800–2000 gm. in weight were used. They were submitted, 6 weeks before the experiments, to a preliminary operation (7) to develop collateral circulation about the liver. At the end of this period, and following a fast of 78 hours, with access to water, hepatectomy was done under ether anesthesia by a method already described (7), and two short, paraffined, glass cannulae were put in the left carotid artery and the left external jugular vein. Each cannula, already filled with 10 per cent sodium citrate solution, had on its free end a tightly fitting bit of rubber tubing a centimeter in length and after it had been filled with isotonic sodium citrate solution (3.8 per cent), it was plugged with a short bit of glass rod. The plugs drove some of the citrate solution into the lumina of the vessels—thereby preventing clotting about them. A sterile vaseline dressing kept the vessels and surrounding structures moist.

In several preliminary experiments these cannulae were not put in place until the transfusions were about to be done. In such instances unavoidable traction on the cervical nerves in animals already in the second stage of liver insufficiency caused depression of the blood pressure and in one instance death. Thereafter the cannulae were always inserted at the time of the original operation.

It was planned to perform "liver transfusions" only after the second stage was clearly manifest in the liverless animal. As a period of 12–16 hours usually elapsed before symptoms of second stage liver insufficiency developed, hepatectomy was usually done in the evening.

The animals were given warm 25 per cent glucose by mouth and left in a warmed cage for the night. The following morning they appeared quite normal. Examination showed the pupillary and musculo-motor reflexes, pulse, temperature and respiration to be normal. A blood specimen of 0.2 cc. was taken for blood sugar determination, but, without waiting for the finding, warmed glucose, 10–15 cc., was intravenously injected in isotonic solution (5.4 per cent). At regular intervals thereafter the injections were repeated. Frequent blood sugar determinations by the Hagedorn-Jensen (10) method served as a guide to regulate the amounts to be given to keep the blood sugar concentration well above normal at all times. At intervals the blood pressure was determined through the carotid cannula.

To maintain the temperature of the animals, they were kept on a warm pad and roofed over by a wire caging covered with cotton wadding. Besides the pad a silvered electric light bulb kept up the warmth in the caging. As at least one observer was in constant attendance upon the animals, the temperature was regulated by turning the light on or off.

From time to time the pupillary and musculo-motor reflexes were examined. At these times, before the second stage symptoms of liver deprivation had begun they were normal, and the pulse, respiration and temperature, recorded at intervals of a few minutes through all the experiments, were normal too.

While the liverless rabbit was still in the first stage, blood specimens from four male rabbits of about the same size were taken and examined for cross-agglutination with the blood of the liverless animal. From amongst the four, three donors with compatible blood were readily selected. Incompatibility is rare in rabbits.

The approach of the second stage after hepatectomy was usually first detected by a change in the animals' posture. During the first stage hepatectomized rabbits receiving the necessary injections of dextrose sit as do normal animals, with elevated head and the paws drawn well under the body. At the approach of the second stage one notices a drooping head and the paws tending to sprawl apart. The animals seemed startled when touched and soon showed ataxia if placed on the floor and allowed to move about.

With the development of these early signs in the liverless animal with blood sugar artificially maintained at a high level, two of the selected donors were bled 30–35 cc. The blood was defibrinated and placed in a water bath at 39°C. When a short time later the signs of the second stage of liver insufficiency had become clearly manifest in the liverless animal a third, freshly fed donor rabbit was anesthetized with ether and injected intravenously with 5 cc. of 1 per cent heparin solution. Paraffined cannulae, filled with the defibrinated blood and plugged, were swiftly placed in the portal vein above the juncture with the splenic vein and also in the vena cava just above the entrance of the renal vein. A third cannula was inserted into the bile duct after tying and cutting the cystic duct. The thorax was then opened and a ligature rapidly thrown about the inferior vena cava above the diaphragm. The liver, with cannulae in place in the extrahepatic portions of its vessels, was removed together with the portion of diaphragm surrounding the vena cava. The whole was immediately submerged in a bath of warm paraffin oil at 39°C. contained in a soft basin of rubber dam tissue fixed within the rim of an iron ring-stand. At once thereafter the cannulated carotid artery of the liverless rabbit was connected with the portal vein cannula of the liver preparation, and the jugular was hitched to the inferior vena cava. This was done by means of short paraffined rubber tubes which, together with the cannulae, were filled with defibrinated blood and clamped. The tube leading from the carotid artery bore a side arm connecting with a mercury manometer for blood pressure readings. 3 cc. of 1 per cent heparin solution was added to 15 cc. of blood from one of the donor rabbits and the tube filled with this and clamped between the manometer and the liver preparation. Now, 25 cc. of the blood of the first or second donor rabbit was slowly injected through the rubber tubing beyond the clamp in the direction of the portal vein of the liver preparation, together with 5 cc. of 1 per cent heparin solution. This was done to charge the liver preparation with blood, thus preventing subsequent loss of blood from the liverless animal into it. No fall in blood pressure of the hepatectomized animal resulted when heparin was injected first into the liver preparation and distributed to the blood in this way. The clamp on the vena cava-jugular vein connection was released and at once a flow of blood occurred from the liver toward the hepatectomized rabbit. The remaining clamp on the carotid artery-portal vein connection was next removed and circulation through the excised liver commenced. As result the arterial blood passed through the portal vein to the liver lobules, and reaching the vena cava by way of the hepatic vein, entered the liverless rabbit once again. The upper end of the vena cava had been ligated and so too had the hepatic artery. Excessive flow of blood from the carotid artery, with resultant distention of the liver with blood, was prevented by partial occlusion of the tube attached to the carotid cannula. The temperature of the oil bath containing the liver was maintained at 38–38.5°C. by constantly stirring in additions of paraffin oil at 40.5°C. and draining an equal quantity from the basin. The liverless rabbit remained on the warm pad under the canopy of cotton wadding. Pulse, temperature and respiratory rate were constantly observed.

Blood flow was usually visible in the cannulae but at intervals the occurrence of flow through the liver was confirmed by pinching the tube leading to the jugular vein. Under these circumstances the livers filled with bright blood in about 20 seconds when full flow from the artery was permitted, and emptied in 40 seconds when it was cut off. Bubbles of lymph were seen escaping from the cut lymphatics of the liver, under the oil, and bile was copiously secreted, as shown by the flow from the cannula in the bile duct. In the four experiments of this nature the livers were transfused from 1½ to 4 hours. To terminate the transfusion the connecting tubes were simply cut and plugged, avoiding all traction on the cervical nerves. A blood specimen of the hepatectomized animal was then taken at once for sugar determination and an intravenous injection of warmed isotonic glucose was given without awaiting the blood sugar findings. The pupillary and musculomotor reflexes were examined and the animal was observed for ataxia, weakness, blindness and other symptoms of second stage liver insufficiency. It was kept on the warm pad until death occurred, given ample glucose, and pulse, temperature and respiration were observed at short intervals. Careful autopsies were performed to determine whether hemorrhage, insufficient collateral circulation or any cause of death could be found, other than absence of the liver. Blocks were taken from the different lobes of the donor liver. Invariably the sections from these were completely normal in appearance.

The transfusion of the blood of the liverless animals through normal livers produced no clinical improvement whatever, although the functional integrity of the donor liver was evidenced both by the formation of much normal looking bile during the experiments and by the lack of histological change of the hepatic tissue. In each of the experiments some 6–8 cc. of clear green bile was obtained from the cannula in the bile duct. Furthermore, the flow of blood through the transfused livers was always excellent. As soon as the liver transfusion was begun the donor liver became bright red and swollen; but the blood coming from it was distinctly darker than that entering. Partial occlusion of the recipient's carotid artery produced prompt reduction in the liver's size and a change in color to a darker hue.

Cross-Transfusions between Normal and Liverless Animals

Since "liver transfusion" failed to ameliorate or reverse the symptoms of advanced liver insufficiency it seemed wise to resort to cross-transfusions between liverless animals and intact normal ones. This procedure introduces many influences not present in a "liver transfusion;" and if it were carried out soon enough after hepatectomy there should be no reason for the liverless rabbit to develop any insufficiency. We purposely delayed it until the second stage of liver insufficiency had set in. Under these circumstances an opportunity was provided for amelioration through an elimination of toxic substances by way of the intact liver and the excretory organs of the donor. One was also afforded for the observation of possible effects of the cross-transfusions upon the donors, from which one might infer the accumulation of toxic substances in the blood of the liverless rabbits. In many ways the symptoms of the rabbit dying of liver insufficiency suggest that there exists a toxic depression of the central nervous system (4). To explore this possibility some of the cross-transfusions were so carried out as to prevent the dilution of the recipient's blood before it should reach the central nervous system of the donor. In these, as will be described below, the blood leaving each animal from a cannula in the proximal end of the carotid artery entered the circulation of the other through a cannula situated in the distal end of the carotid. In the ordinary experiments cross-transfusions were performed from the carotid artery of one animal to the jugular vein of the other and *vice versa.*

Control Experiments.—Four cross-transfusions between pairs of equal sized normal rabbits were performed under ether anesthesia. In two experiments, after testing the bloods of the prospective partners for compatibility, paraffined cannulae were inserted in the left carotid arteries and left jugular veins of each animal and connected by short paraffined rubber tubes 3 cm. in length in such a way that the carotid blood of one animal flowed into the jugular vein of the other. In the other experiments two cannulae were placed in the left carotid artery of each of the animals and connected by rubber tubes in such a way that blood flow from the proximal end of the carotid artery of each one passed into the distal end of the carotid of the other. To prevent clotting in the cannulae prior to the transfusion they were filled with heparinized blood and stoppered as in the previous work. 5 minutes before each experiment was begun both animals received intravenously 3–5 cc. of 1 per cent heparin solution. The cross-transfusions were allowed to continue 1, 2, 3 and 4 hours respectively. No untoward symptoms developed. Only insignificant changes in pulse and respiratory rate were observed. In one of the experiments the effect of bleeding one animal into the other and *vice versa* was repeatedly tried, by temporarily clamping the carotid artery of one. When the blood pressure of the bled animal fell to about 10 mm. of mercury that of the other rose but 15–20 mm. above its "normal" under the conditions. Both pressures returned to the previous level when the cross-flow of blood was again permitted.

Cross-transfusions were next carried out between normal rabbits and liverless ones in the second stage of liver insufficiency. The liverless animals at times showed slight improvement, perhaps owing to better circulation of the blood, for the skin often became more pink and warm, but, though the cross-transfusions were continued, the animals lapsed rapidly into their previous state.

Methods and Technique

Rabbits of 1800–2000 gm. were submitted to a preliminary operation (7) to develop collateral circulation about the liver, and later, under ether anesthesia, the liver was removed and two cannulae inserted in the vessels of the neck. Both were placed in the same carotid artery in some experiments, one directed toward the heart, the other toward the head; and in other experiments one in the carotid artery, toward the heart, the other in the jugular vein. They were filled with 10 per cent sodium citrate solution and closed off with pieces of glass rod as usual.

Save where special mention is made the further postoperative treatment of the liverless animals was as in the previous work. Warmed glucose was given by mouth the first night after operation and on the following day intravenously, frequent blood sugar determinations serving as guide to the amounts to be given. As routine, but more frequently than in the instances used for "liver transfusions," the blood pressure of the animals was taken from the carotid cannula,—at hourly or half hourly intervals,—to determine the pressures at which the later cross-transfusion should be done.

For the transfusion two donor rabbits of about the same size as the liverless recipient were selected by cross-agglutination tests for compatibility. From one 40–50 cc. blood was taken with light ether anesthesia from the carotid artery, aseptically defibrinated and kept in a water bath at 39°C., ready for use as will be described below. The other donor was lightly anesthetized with ether and two cannulae were placed in the neck vessels, the location depending upon the type of cross-transfusion to be done.

After hepatectomy about half the rabbits passed into the second stage of liver insufficiency without significant changes in blood pressure as measured from time to time by a mercury manometer brought into connection with the cannula in the carotid artery.

The others showed a marked fall prior to the onset of the second stage. As the experiments demanded postponement of the transfusions until the animals were manifestly in this stage, and as we did not wish to mistake an ordinary improvement in blood pressure resulting from transfusion for an improvement in general condition due to relief from the hepatectomized condition, the liverless animals with low blood pressure were given injections of the defibrinated blood in sufficient amount to maintain the blood pressure until the symptoms of the second stage had fully developed. At times 25–40 cc. was required, given slowly at intervals, in 3–4 cc. amounts. When the second stage was fully established in the recipient, both animals were given 5 cc. each of heparin solution (10 mg. per cc.), and cross-connections were made with the cannulae by means of short rubber T tubes in such a way that blood flow from the proximal end of the carotid artery of each passed into the distal end of the other and so toward the brain, or, in the instances in which the jugular vein had been cannulated, from carotid artery to vein. Mercury manometers were attached to each of the tubes connecting the cannulae. These afforded continuous readings of the pressure at which blood flowed from one animal to the other. By clamping the tube on either side of the manometer by-pass, the blood pressure in the carotid of either animal could be obtained. The tubes leading to the manometers also carried a short side arm attached to a greased Luer syringe through which blood could be added to or recovered from the circuit at will. Immediately before beginning cross-transfusion the blood pressure of the recipient was brought up to that of the donor, when it was somewhat lower, by the addition of as much compatible defibrinated blood as necessary. The blood, already taken from one of the donors, was given through a syringe to the donor of the cross transfusion and that animal was allowed to bleed an equal amount into a second syringe. This blood was in turn given to the recipient. In this way we avoided direct injection of defibrinated blood into the sick recipient, yet without changing the donor's blood pressure allowed the latter to furnish blood enough to the recipient to equalize the pressures in the animals. The cross-transfusion was then begun and the flow of blood adjusted thereafter by partial occlusion of the connecting tubes, so that the blood pressure of the liverless recipient remained throughout the experiment at approximately the same level as previously, or very slightly higher.

It was found by repeated determinations in the majority of experiments that the blood pressure of each animal at the proximal end of the carotid artery averaged about 5 mm. of mercury more than the transfusion pressure, that is to say the reading of the manometer without obstruction to the blood flow between animals.

The cross-transfusions were allowed to run for 2–4½ hours. At frequent intervals tests for flow in the connecting tubes were made by means of the greased Luer syringes. Simultaneous obstruction of the afferent blood flow to each animal close to the distal carotid (or jugular vein) cannulae caused the efferent streams of blood to pass into the syringes. By releasing the obstruction and in turn preventing efferent flow from both animals the material in the syringes could be expelled by way of the distal cannulae. In the instances in which jugular cannulations were used the jugular veins at each end of the venous cannula showed a bulbous swelling which disappeared at once when the tubes leading to them were pinched. Furthermore blood flow was usually visible in the cannulae.

As the transfusions continued the blood pressures of donor and recipient usually fell slightly. At intervals of approximately 20 minutes, 2½–4 cc. of a 1 per cent heparin solution was injected in small doses into the tube leading to the donor. Transient falls in blood pressure resulted but were recovered from rapidly. Occasionally the blood pressure of one animal or the other would increase and there must have resulted some surge of blood from the one with higher pressure to the other. But pressure equilibrium was always soon re-established.

In two of the experiments the untouched carotid artery of the donor rabbit was ligated after the cross-transfusion had run for half an hour. This rendered the cerebral circulation of the donor almost wholly dependent upon blood supplied by the liverless rabbit.

As in the "liver transfusions" so in these the cross-flow of blood was terminated, when desired, by cutting and plugging the connecting tubes. Thus all traction on cervical nerves was avoided. As soon as a cross-transfusion was discontinued the liverless animal was given glucose, its corneal and musculo-motor reflexes were tested and it was examined for ataxia.

In the twelve experiments, five of which were wholly free from technical errors, the liverless rabbits showed no improvement. During the cross-transfusions the pulse rate slowed slightly, as one would expect with the advancing second stage of liver insufficiency. Immediately after the transfusions the animals showed no amelioration of the symptoms of the second stage. The muscular weakness, ataxia, apparent blindness and diminution of the corneal reflexes were usually more pronounced than before the transfusion. In a few instances gasping respiration, present before the transfusion, ceased for the first half hour of blood flow but began again before the transfusion was finished. In general it can be said that the second stage of hepatic insufficiency progressed during the cross-transfusion itself, although its progress was usually far more rapid after the procedure.

In three of the five experiments the liverless animals entered the second stage with normal or almost normal blood pressure. In two instances abnormally low pressures existing an hour or two before cross-transfusion were rectified by injections of blood and further raised when the cross-transfusion was begun. The improvement was slight and short lived, however, and the phenomena of the second stage of liver insufficiency became more marked during the transfusion period,—this despite the fact that the pale skin of these recipients often became pink and that in one instance the blood pressure was raised from 40 mg. of mercury to 100. Clearly the late course of events after liver deprivation, that which leads to death, is not averted or even importantly delayed by cross-transfusion. We have rigorously ruled out all instances in which slips in technique or pathological conditions might have affected the results.

DISCUSSION

The work here detailed has shown the late changes occurring in liver insufficiency to be irreversible. What can one say of the origin of these lethal changes arising in the animal deprived of the liver?

It is evident that the second stage of liver insufficiency does not result merely from an overwhelming accumulation of toxic substances in the organism, which might be eliminated or neutralized by a liver if one were supplied. It is known that the products of incomplete metabolism accumulate in the blood of the liverless animal. Bollman, Mann and Magath (11) have reported the presence of increased amounts of uric acid and a probable increase in ammonia (12) in the blood of dogs deprived of the liver. Work already reported from this laboratory has confirmed these uric acid findings in rabbits suffering from pronounced liver insufficiency (6). Doubtless other products of incomplete metabolism accumulate in the blood of the animals as well. Both in the "liver transfusion" and cross-transfusion experiments there was ample opportunity for the donor liver to destroy or excrete toxic substances in the blood if such had been present. Furthermore in the cross-transfusion experiments there must have occurred a dilution of any toxic substances, present in the recipient's blood, by admixture with the donor's blood. Despite these favorable conditions no improvement was observed in the condition of the liverless animals.

A toxic product accumulating in the blood of a liverless rabbit in amounts sufficient to kill might be expected to register some deleterious effect upon a normal animal through which the entire blood bulk, of the liverless one was suddenly circulated by transfusion. But no such effect was observed upon the donor rabbits in our cross-transfusion experiments. The animals serving as donors were allowed to recover from the anesthetic and carefully observed. They exhibited no abnormal symptoms whatever, even in the instances in which the hepatectomized rabbit's blood was allowed to run cephalad into the carotid artery of the donor, thus coursing through the brain and central nervous system of the latter before reaching the liver.

The symptoms of the second stage of liver insufficiency cannot be due to the mere lack of some essential substance, hormone or what not, which can be supplied from without at a late stage, as can sugar to prevent early death from deprivation of the liver. Previous work on hepatectomized animals (1–9) has not demonstrated a lack of essential substances other than glucose, but such work has not sufficed to exclude the possibility. The present work has made plain the fact that the normal organism cannot supply anything to the liverless one that will do away with or even ameliorate the second stage phenomena after hepatectomy, once these have appeared. But perhaps the second stage had failed to appear until the lack became so great that irremediable damage had been done. Against the validity of this improbable assumption there is evidence, but unfortunately no proof. The liverless animal at the time of the onset of the second stage is normal in appearance and our histological examinations at autopsy, even in the untreated liverless animal, show no definite degenerative changes in the central nervous system or elsewhere. Yet cross-transfusion fails to prolong life in these animals. The outlook for prolonging the life of human beings with severe liver insufficiency by supplying "biologicals" is not a promising one.

An accumulation of toxic substances or a lack of something essential there may be in the hepatectomized organism but if so this lack or accumulation has resulted in irreversibly damaging changes by the time the second stage has set in.

Was there an accumulation of toxic products which became fixed upon the tissues? The foregoing discussion has concerned itself chiefly with the possible presence or lack of substances freely circulating in the blood of the liverless animals. A toxic substance present in the blood in minute quantity might conceivably be continually removed or rendered harmless by the liver, but become fixed within the tissues on the absence of the organ, causing irreversible damage. Such a happening would account for the failure of cross-transfusion to cause any betterment. An irreversibility of the changes of liver deficiency as manifested by the second stage phenomena has been the invariable finding in our work.

Yet another possibility remains. Perhaps the liver by means of minute amounts of some substances controls the activity of other organs, and when it is lacking, some irreversible change takes place.

SUMMARY

The symptoms of advanced liver insufficiency, in the hepatectomized rabbit, are irreversible. That is to say, cross-transfusions between liverless rabbits and normal ones fail to ameliorate the symptoms of liver deprivation once they are established. The normal rabbits show no symptoms suggesting that toxic substances have accumulated in the blood of the hepatectomized ones.

BIBLIOGRAPHY

1. Mann, F. C., *Am. J. Physiol.*, 1921, **55,** 285.
2. Mann, F. C., and Magath, T. B., *Arch. Int. Med.*, 1922, **30,** 73.
3. Mann, F. C., and Magath, T. B., *Arch. Int. Med.*, 1922, **30,** 171.
4. Mann, F. C., *Medicine*, 1927, **6,** 419.
5. Mann, F. C., *Ergebn. Med.*, 1925, **24,** 379.
6. McMaster, P. D., and Drury, D. R., *J. Exp. Med.*, 1929, **49,** 745.
7. Drury, D. R., *J. Exp. Med.*, 1929, **49,** 759.
8. Drury, D. R., and McMaster, P. D., *J. Exp. Med.*, 1929, **49,** 765.
9. McMaster, P. D., and Drury, D. R., *Proc. Soc. Biol. and Med.*, 1929, **27,** 47.
10. Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, Berlin, 1923, **135,** 46.
11. Bollman, J. L., Mann, F. C., and Magath, T. B., *Am. J. Physiol.*, 1925, **72,** 629.
12. Bollman, J. L., Mann, F. C., and Magath, T. B., *Am. J. Physiol.*, 1926, **78,** 258.

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SYNTHESIS OF THE PHOSPHORIC ESTERS OF HYDROXYAMINO ACIDS

III. Resolution of *d,l*-Serinephosphoric Acid and Synthesis of *l*-Hydroxyprolinephosphoric Acid

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In previous communications of this series[1, 2] the synthesis of the phosphoric esters of two hydroxyamino acids (namely natural tyrosine and *d,l*-serine) was reported. It was desired to prepare the phosphoric esters of the other naturally occurring hydroxyamino acids.

The present communication contains a report on the synthesis of the phosphoric ester of the natural *l*- (levo-)hydroxyproline. This substance was prepared by the method which led to the formation of the ester of *d,l*-serine.

At the same time, we wish to state that the method was not applicable to the synthesis of the phosphoric esters of either hydroxyaspartic or hydroxyglutamic acids. In fact, up to the present, we have failed to prepare either of these esters by any other method of phosphorylation which we have applied.

The present paper also contains a report on the resolution of *d,l*-serinephosphoric acid to give dextro-serinephosphoric acid, the maximum rotation of which was found practically identical with that of the naturally occurring substance. The purity of the substance described by Lipmann and Levene[3] is thus definitely established.

Occasion was also taken to convert the dextro- (*d*-)phosphoser-

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[1] Levene, P. A., and Schormüller, A., *J. Biol. Chem.*, **100**, 583 (1933).

[2] Levene, P. A., and Schormüller, A., *J. Biol. Chem.*, **105**, 547 (1934).

[3] Lipmann, F. A., and Levene, P. A., *J. Biol. Chem.*, **98**, 109 (1932).

ine into levo-phosphoglyceric acid. The ease with which this reaction is accomplished indicates that the same reaction may occur in the living organism and, in view of the part played by phosphoglyceric acid in carbohydrate metabolism, a new physiological significance may be attributed to phosphoproteins.

Incidentally, mention may be made of the use of Raney's catalyst for the synthesis of aminomalonic ester and hydroxyglutamic acid. In the latter case the method of Harington and Randall[4] is more advantageous from the view-point of yield; on the other hand, by Rancy's method, the isolation of the intermediate product—the dihydropyrazine derivative—was accomplished without difficulty.

EXPERIMENTAL[5]

Dextro-Serinephosphoric Acid—The *d,l*-serine required for the preparation of *d,l*-serinephosphoric acid was prepared essentially according to the method of Leuchs and Geiger.[6] However, instead of the chloroacetal, the bromoacetal (obtained from paraldehyde with bromine) was treated with sodium ethylate on the steam bath,[7] thereby producing the ethoxyacetal by a much simpler process and with a very good yield. The *d,l*-serine (200 gm.) was phosphorylated[2] in portions of 8 gm. and yielded 106 gm. of the barium salt of *d,l*-serinephosphoric acid.

Two portions of the barium salt (50 gm. each) were each suspended in 400 cc. of water. Sulfuric acid was added in slight excess and the mixture vigorously shaken mechanically during 1 hour. The solutions were combined, filtered, and quantitatively freed from barium and sulfate ions. The clear solution was made faintly alkaline by addition of a concentrated solution of brucine in methyl alcohol. The excess brucine was then removed by extraction with chloroform and the aqueous layer evaporated under diminished pressure to a thick syrup.

The product was dissolved in 750 cc. of boiling methyl alcohol and the solution cooled in ice water during 2 hours. On filtering through a Buchner funnel, there were obtained 175 gm. of nicely crystallized brucine salt. On concentrating and standing in the refrigerator the mother liquor yielded an additional 40 to 50 gm. of the salt.

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[4] Harington, C. R., and Randall, S. S., *Biochem. J.*, **25**, 1917 (1931).

[5] We wish to thank Dr. R. S. Tipson for his kindness in assisting in the preparation of the manuscript.

[6] Leuchs, H., and Geiger, W., *Ber. chem. Ges.*, **39**, 2644 (1906).

[7] Späth, E., *Monatsh. Chem.*, **36**, 4 (1915).

175 gm. of the first crop of brucine salt were recrystallized four times from absolute methyl alcohol and a small sample then converted into the barium salt. The optical rotation of the barium salt, dissolved in 10 per cent hydrochloric acid, was

$$[\alpha]_D^{25} = \frac{+0.75° \times 100}{2 \times 6} = +6.3°$$

After seven recrystallizations, the rotation under the same conditions was +8.6°. After nine recrystallizations, the rotation was constant (+9.4°) and was not changed by further crystallization. The yield was 40.5 gm.

Dried under diminished pressure at 60°, the dibrucine salt had the following composition.

5.940 mg. substance: 0.382 cc. N_2 (757 mm. at 26°)
4.190 " " : 8.980 mg. molybdate
$C_{49}H_{60}O_{14}N_5P$.* Calculated. N 7.20, P 3.19
973.5 Found. " 7.31, " 3.11

* In our former paper[8] the formula and calculated values for the dibrucine salt should be corrected.

On heating, the salt sinters at 100° and decomposes[8] around 130°. The barium salt, obtained from the brucine salt with barium hydroxide, had the following composition after drying at 110°.

10.580 mg. substance: 0.387 cc. N_2 (762 mm. at 26°)
3.472 " " : 23.180 mg. molybdate
$C_3H_6O_6NPBa$. Calculated. N 4.36, P 9.65
320.4 Found. " 4.18, " 9.69

For the determination of the specific rotation, a 6 per cent solution of the barium salt in 10 per cent hydrochloric acid was used.

$$[\alpha]_D^{25} = \frac{+1.13° \times 100}{2 \times 6.0} = +9.4°$$

Calculated as the free acid, the rotation[3] was

$$[\alpha]_D^{25} = \frac{+1.13° \times 100}{2 \times 3.47} = +16.3°$$

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[8] Levene, P. A., and Schormüller, A., *J. Biol. Chem.*, **103**, 537 (1933).

Phosphate of l-Glyceric Acid from d-Serinephosphoric Acid—To a suspension of 4.8 gm. of finely powdered barium salt of *d*-serinephosphoric acid in 100 cc. of water 2.7 gm. of concentrated sulfuric acid were added and the mixture was vigorously shaken during 30 minutes. To this suspension a concentrated aqueous solution of 2.3 gm. of pure crystallized barium nitrite ($Ba(NO_2)_2 \cdot H_2O$) was slowly added during 1 hour, being cooled in ice throughout the operation. The mixture was then allowed to stand overnight at room temperature.

A small portion of the liquid was now concentrated under diminished pressure, rendered faintly alkaline by addition of barium hydroxide, and filtered through charcoal. On rapidly heating the solution to boiling, the barium salt was precipitated and was filtered off while hot. After reprecipitating with alcohol from an aqueous solution made faintly acid to Congo red[9] the substance still contained 1.5 per cent of nitrogen.

Therefore, the larger part of the reaction liquid was treated once more with 1.2 gm. of sulfuric acid and 2.3 gm. of barium nitrite as described above. Finally, after standing for 3 to 4 hours, the barium salt was prepared. The yield was 1.4 gm.

The substance was dried at 110° for analysis and had the following composition.

5.005 mg. substance: 2.120 mg. CO_2 and 0.980 mg. H_2O
4.550 " " : 29.900 " molybdate
40.470 " " : 27.820 " $BaSO_4$

$C_3H_5O_7PBa$.	Calculated.	C 11.20, H 1.55, P 9.64, Ba 42.75
321.4	Found.	" 11.55, " 2.19, " 9.54, " 40.45

For the determination of the specific rotation, Vogt's[9] method was followed. 0.4018 gm. of the barium salt was dissolved in 2.5 cc. of N sulfuric acid and immediately neutralized with 2.5 cc. of N sodium hydroxide. The rotation was

$$[\alpha]_D^{25} = \frac{-1.00° \times 100}{2 \times 8.036} = -6.2°$$

Calculated as the free acid, the rotation was

$$[\alpha]_D^{25} = \frac{-1.00° \times 100}{2 \times 4.65} = -10.7°$$

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[9] Vogt, M., *Biochem. Z.*, **211**, 1 (1929). Neuberg, C., Weinmann, F., and Vogt, M., *Biochem. Z.*, **199**, 248 (1928).

After the addition of a few drops of concentrated hydrobromic acid the rotation changed,[9] and after standing for 3 days it was

$$[\alpha]_D^{25} = \frac{+0.39° \times 100}{2 \times 8.036} = +2.4°$$

Dextro-serine, converted into glyceric acid, yields the levo acid.[10]

Levo-Hydroxyprolinephosphoric Acid—Phosphorus pentoxide (4.0 gm.) was dissolved in 40 gm. of 100 per cent phosphoric acid. To this solution were added 5.2 gm. of pure hydroxyproline (very finely powdered) and the mixture was thoroughly stirred. After standing in a desiccator during 50 to 60 hours, finely chopped ice and then 2 liters of ice water were added. 180 to 200 gm. of crystallized barium hydroxide and a little phenolphthalein were now added and the mixture was vigorously stirred until it became pink in color. The barium phosphate was removed by centrifuging and the precipitate well stirred with about 1 liter of water and filtered through a thin layer of charcoal. The combined solutions were rendered neutral by addition of a few drops of hydrobromic acid and then concentrated under diminished pressure at 40° to a volume of 150 to 200 cc. The solution was made faintly alkaline with barium hydroxide, filtered through charcoal, and alcohol was added until the concentration of alcohol was about 60 per cent. The thick precipitate was readily separated by centrifuging. After washing successively with 50 per cent alcohol, 80 per cent alcohol, alcohol-ether 1:1, and anhydrous ether, the barium salt was filtered off and dried. Yield 4.3 gm.

The barium salt is a very fluffy, white powder which is easily soluble in cold water. On heating the aqueous solution, the barium salt separates as a compact, crystalline precipitate, which consists of imperfectly formed feather-like crystals.

For analysis, the salt was dissolved in a little cold water, precipitated by boiling the solution, redissolved in cold water, and finally precipitated with alcohol. After drying at 80° under diminished pressure, it still contains 1 mole of water of crystallization which can be driven off by drying at 130° under a high vacuum during 24 hours.

145.5 mg. substance lost 8.0 mg. H_2O
$C_5H_8O_6NPBa \cdot H_2O$. Calculated. H_2O 4.9
364.5 Found. " 5.5

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[10] Fischer, E., and Jacobs, W. A., *Ber. chem. Ges.*, 40, 1068 (1907).

The barium salt, dried at 80°, had the following composition.

7.190 mg. substance: 4.185 mg. CO_2 and 2.090 mg. H_2O
8.696 " " : 0.297 cc. N_2 (754 mm. at 27.5°)
4.004 " " : 23.450 mg. molybdate
0.2200 gm. " : 0.1405 gm. $BaSO_4$
$C_5H_8O_6NPBa \cdot H_2O$ (364.5)
Calculated. C 16.48, H 2.7, N 3.84, P 8.52, Ba 37.64
Found. " 16.06, " 3.2, " 3.85, " 8.51, " 37.58

For the determination of the specific rotation, the barium salt was dried at 130° and dissolved in 10 per cent hydrochloric acid.

$$[\alpha]_D^{25} = \frac{-1.60^\circ \times 100}{2 \times 6.0} = -13.3^\circ$$

Calculated as the free acid, the rotation was

$$[\alpha]_D^{25} = \frac{-1.60^\circ \times 100}{2 \times 3.656} = -21.9^\circ$$

Brucine Salt of Hydroxyprolinephosphoric Acid—3.6 gm. of the barium salt of the hydroxyprolinephosphoric acid were dissolved in a little water and the barium quantitatively removed by the addition of about 10 cc. of 10 per cent sulfuric acid. To the aqueous solution a solution of brucine in methyl alcohol was added until the mixture showed a distinctly alkaline reaction to litmus. The excess of brucine was extracted with chloroform and the aqueous layer evaporated to dryness under diminished pressure at 40°.

The residue was dissolved in a little hot butyl alcohol (about 75 per cent) and allowed to stand in the refrigerator overnight. The brucine salt crystallized out in rosettes of large needles.

These crystals were dried over phosphoric anhydride at room temperature and then on further drying at 130° in a high vacuum during 30 hours, 5 moles of water were lost. The dry substance had an indefinite melting point between 180–183°. It had the following composition.

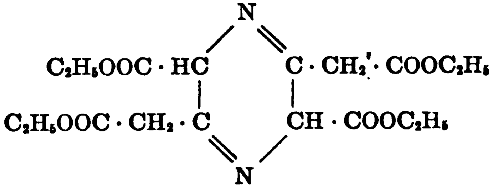
7.965 mg. substance: 0.483 cc. N_2 (756 mm. at 25°)
3.810 " " : 9.500 mg. molybdate
$C_{51}H_{62}O_{14}N_5P$. Calculated. N 7.01, P 3.11
999.5 Found. " 6.90, " 3.60

Diethyl Ester of Aminomalonic Acid[11]—25 gm. of isonitrosomalonic ester (purified by distillation under diminished pressure) were dissolved in 45 gm. of absolute alcohol, 5 gm. of Raney's nickel catalyst were added, and the mixture was shaken with hydrogen at a pressure of 40 pounds per sq. inch. After 4 to 5 hours, the absorption of hydrogen ceased. The catalyst was removed by centrifuging and the alcohol evaporated off under diminished pressure. The syrup was mixed with 250 cc. of anhydrous ether and saturated with dry hydrogen chloride. After standing for 2 hours in the refrigerator, the thick paste was filtered through a Buchner funnel and the product washed with dry ether. Yield 18 to 21 gm.

For analysis it was dissolved in a small amount of absolute ethyl alcohol containing 1 per cent of dry hydrogen chloride and precipitated by the addition of warm ether. The melting point was 170°.

10.610 mg. substance: 0.608 cc. N_2 (759 mm. at 24°)
5.746 " " : 3.800 mg. AgCl
$C_7H_{14}O_4NCl$. Calculated. N 6.62, Cl 16.75
211 Found. " 6.57, " 16.36

Isolation of Diethyl Ester of 2,5-Dicarbethoxy-Dihydropyrazine-3,-6-Diacetic Acid (I) in Preparation of Hydroxyglutamic Acid[4]



I

25 gm. of ethyl acetone dicarboxylate in 75 cc. of absolute alcohol were shaken in the presence of Raney's nickel catalyst with hydrogen at a pressure of 40 pounds per sq. inch until the absorption had ceased (3 to 4 hours) and the solution was then centrifuged in order to remove the catalyst. After standing overnight in a well stoppered bottle at room temperature, the brown liquid was cooled

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[11] Piloty, O., and Neresheimer, J., *Ber. chem. Ges.*, **39**, 514 (1906). Putochin, N. J., *Ber. chem. Ges.*, **56**, 2213 (1923).

in ice for several hours. During this time, a thick paste of golden-yellow needles formed.

The ester crystallized from absolute alcohol in long, bright yellow needles having a melting point of 124°. For analysis, it was twice recrystallized from absolute ethyl alcohol and dried at 60° under diminished pressure. It had the following composition.

4.465 mg. substance: 8.870 mg. CO_2 and 2.580 mg. H_2O
7.960 " " : 0.515 cc. N_2 (759 mm. at 25°)
$C_{18}H_{26}O_8N_2$. Calculated. C 54.24, H 6.6, N 7.04
398.2 Found. " 54.17, " 6.5, " 7.40

Conversion of (I) into 3,6-Dimethylpyrazine—The method given by Harington and Randall[4] was followed exactly. The dimethylpyrazine was purified by isolation of the mercuric chloride compound. This was converted into the picrate, which crystallized from absolute alcohol in long lemon-yellow needles and had a melting point of 157°.

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MONOACETONE *d*-XYLOKETOSE

A PRELIMINARY NOTE

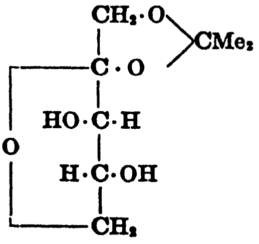
BY P. A. LEVENE AND R. STUART TIPSON

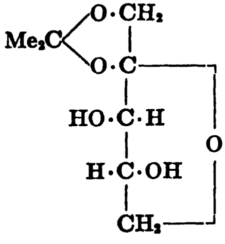
(*From the Laboratories of The Rockefeller Institute for Medical Research*)

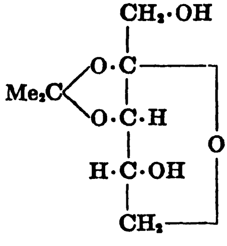
(Received for publication, July 2, 1934)

Whereas in the case of hexoses both the aldoses and the ketoses have been the subject of extensive investigation, in the case of pentoses the *keto* forms have attracted practically no attention, since they are difficultly accessible either as natural products or by methods of synthesis. Of the four possible forms only one has been discovered in nature—the *keto*xylose isolated from urine, in cases of pentosuria, by Levene and La Forge.[1] The *d* form of this sugar was recently synthesized by Schmidt and Treiber[2] and a detailed study of its properties and of its derivatives is now being undertaken in our laboratory. The present note deals with the preparation of the monoacetone derivative.

d-Xyloketose was condensed with acetone in the presence of sulfuric acid and anhydrous copper sulfate to give a crystalline monoacetone derivative which does not exhibit mutarotation in water. It therefore presumably has one of the three following structures.







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[1] Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, **18**, 319 (1914).

[2] Schmidt, O. T., and Treiber, R., *Ber. chem. Ges.*, **66**, 1765 (1933).

Investigation of its precise structure (and of that of the glycosides of *d*-xyloketose) is now in progress.

EXPERIMENTAL

Preparation of Monoacetone d-Xyloketose—*d*-Xyloketose was prepared from *d*-xylose as described by Schmidt and Treiber.[2] 2 gm. of pure xyloketose (regenerated from the *p*-bromophenylhydrazone) were shaken with a mixture of 0.04 cc. of concentrated sulfuric acid, 40 cc. of acetone (analytical reagent), and 4 gm. of anhydrous copper sulfate at room temperature (27°) during 90 hours.

The product was isolated as for monoacetone ribose,[3] giving a pale yellow syrup (yield, 2 gm.) which was purified by distillation under a high vacuum.

The substance boiled at 130–140° (bath temperature) at 0.1 mm. and was collected as a colorless, viscous syrup which quickly crystallized spontaneously to a solid mass.

It had the following composition.

4.673 mg. substance: 8.665 mg. CO_2 and 3.180 mg. H_2O
$C_8H_{14}O_5$. Calculated. C 50.50, H 7.4
Found. " 50.56, " 7.6

The crystalline material was somewhat hygroscopic and had a melting point of 50–52° (with softening at 45°) in a sealed tube.

Its specific rotation was

$$[\alpha]_D^{28} = \frac{-0.10° \times 100}{2 \times 1.930} = -2.6° \text{ (in water, 2 minutes after solution)}$$

The rotation remained unchanged after the addition of 1 drop of concentrated ammonia.

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[3] Levene, P. A., and Stiller, E. T., *J. Biol. Chem.*, **102**, 187 (1933).

[Reprinted from The Journal of Biological Chemistry, September, 1934,
Vol. 106, No. 2, pp. 761-771]

THE SYNTHESIS OF THEOPHYLLINE-5-METHYL-RHAMNOFURANOSIDE

By P. A. LEVENE and IRVING E. MUSKAT*

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, July 12, 1934)

The present communication is one of a series dealing with the synthesis of the naturally occurring nucleotides. For such synthesis it is required to develop methods first, for the preparation of the nucleosides and second, for phosphorylation of the latter in positions (3) or (5) of the ribose chain. Methods for phosphorylation in position (5) of the ribose residue have already been reported from this laboratory.[1,2] Nucleosides derived from pyranoses have been described by several workers but scarcely any attempts have been made to prepare furanosidic nucleosides. Such compounds are of special interest since all the naturally occurring nucleosides so far examined are of this type.

In the present investigation a rhamnofuranose derivative was chosen for condensation with theophylline, the reason being that 5-methyl-triacetyl-rhamnofuranose (VI) is readily converted into a crystalline 1-bromo-5-methyl-2,3-diacetyl-rhamnofuranose (VII). The theophylline condensation product (IX) was obtained in amorphous form but had the correct composition and did not reduce boiling Fehling's solution prior to acid hydrolysis.

The starting material for the preparation of 5-methyl-triacetyl-rhamnofuranose (VI) was the monoacetone rhamnose (I) first prepared by Fischer[3] and shown by Freudenberg[4] to have the furanose structure. The substance prepared by us had all the properties described by Freudenberg; thus the monomethyl monoacetone methylrhamnofuranoside (III) could be converted into a monomethyl rhamnose (IV) which, on oxidation, yielded methoxy lactic acid.

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* National Research Fellow in Chemistry.

[1] Levene, P. A., and Stiller, E. T., *J. Biol. Chem.*, **104**, 299 (1934).

[2] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **106**, 113 (1934).

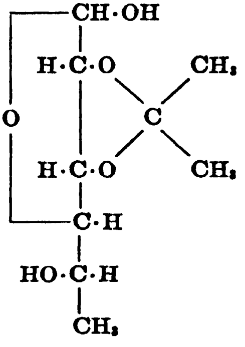
[3] Fischer, E., *Ber. chem. Ges.*, **28**, 1145 (1895).

[4] Freudenberg, K., *Ber. chem. Ges.*, **59**, 836 (1926).

A monotosyl derivative (V) of monoacetone methylrhamnofuranoside (II) was prepared and this was shown not to react with sodium iodide under the conditions which convert primary alcoholic groups into iodides.

Of practical importance for future synthetic work on nucleotides was the possibility of the formation of orthoacetates of furanoses. Evidence was furnished that 5-methyl-rhamnofuranose (IV) forms a 1,2-methylorthoacetyl-3-acetyl-5-methyl-rhamnofuranose (VIII) although the substance has not yet been isolated in pure form.

Incidentally, occasion was taken to compare the advantages of the methylation of the monoacetone methylrhamnofuranose by the method of Muskat[5] with those of the older methods of methylation, the result being favorable.



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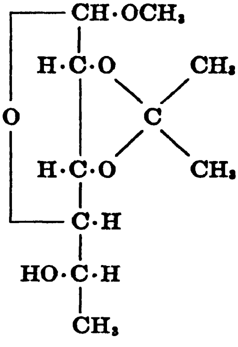
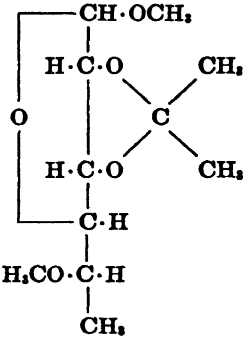
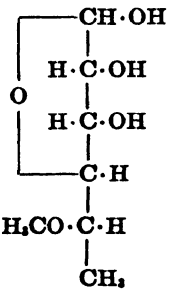


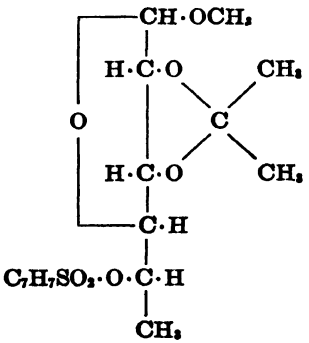
II



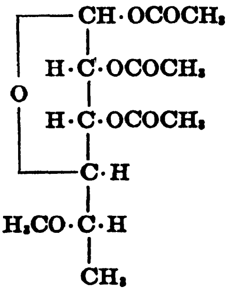
III



IV



V



VI

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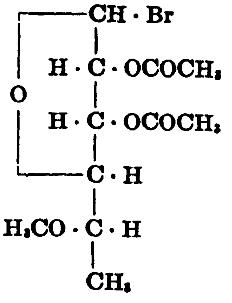
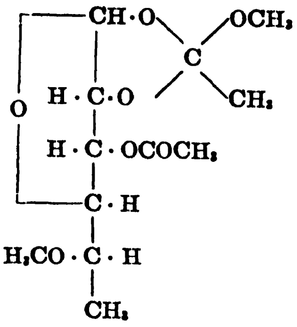
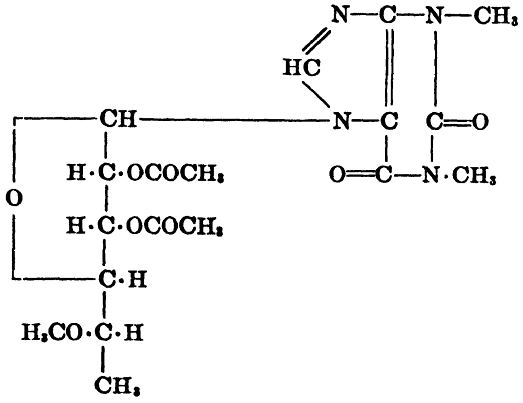
[5] Muskat, I. E., *J. Am. Chem. Soc.*, **56**, 693 (1934).



VII



VIII



IX

EXPERIMENTAL

Preparation of Monoacetone Rhamnofuranose (*I*)—Monoacetone rhamnofuranose was first prepared by Fischer[3] and its structure determined by Freudenberg.[4] Freudenberg showed that it existed in two forms: (1) m.p. 87–89°, $[\alpha]_D$ = 13° → 17.8° (in water); (2) m.p. 79–80°, $[\alpha]_D$ = 10.9° → 17.8° (in water). For this investigation the monoacetone rhamnofuranose was prepared as follows: 10 gm. of anhydrous rhamnose were suspended in 200 cc. of acetone (free of methyl alcohol) containing 0.2 per cent of sulfuric acid. Anhydrous copper sulfate (20 gm.) was added and the mixture was shaken at 37° for 20 hours. The mixture was filtered and the filtrate rendered neutral by shaking with calcium oxide. The calcium sulfate and excess calcium oxide were removed by means of filtration and washed with small quantities of acetone. The filtrate was evaporated under diminished pressure and the residual syrup was distilled. The entire product distilled without decomposition at about 115° under 0.2 mm. pressure. The product crystallized; m.p. 90°. The pure crystals did not reduce Fehling's solution.

Its specific rotation was

$$[\alpha]_D^{20} = \frac{+0.13° \times 100}{2 \times 1.015} = +6.4° \text{ (in water)}$$

$$[\alpha]_D^{20} = \frac{+0.36° \times 100}{2 \times 1.015} = +17.7° \text{ (in water with addition of ammonia)}$$

The substance had the following composition.

5.110 mg. substance: 9.935 mg. CO_2 and 3.610 mg. H_2O

$C_9H_{16}O_5$.	Calculated.	C 52.90, H 7.8
204.1	Found.	" 53.01, " 7.9

Preparation of Monomethyl Monoacetone Methylrhamnofuranoside (III)—Freudenberg[4] first methylated monoacetone rhamnofuranose by the Purdie method. He obtained a product which distilled at 65–67° under 0.5 to 1 mm. pressure, $[\alpha]_D = -32.5°$ (in water).

This preparation was repeated, using 20.4 gm. of monoacetone rhamnofuranose, 100 gm. of methyl iodide, and 120 gm. of silver oxide. The methylation was twice repeated with half the above quantities of methylating agents and the product was isolated as a colorless, mobile syrup which distilled at 65° under 1 mm. pressure. Yield, 16.5 gm. (71 per cent).

Its specific rotation was

$$[\alpha]_D^{20} = \frac{-3.98° \times 100}{2 \times 3.704} = -53.7° \text{ (in methyl alcohol)}$$

The substance had the following composition.

5.179 mg. substance: 10.716 mg. CO_2 and 3.845 mg. H_2O
5.655 " " : 11.260 " AgI

$C_{11}H_{20}O_5$.	Calculated.	C 56.86, H 8.7, OCH_3 26.72
232.2	Found.	" 56.42, " 8.3, " 26.28

Preparation of Monoacetone Methylrhamnofuranoside (II)—Monoacetone methylrhamnofuranoside was prepared by the method described above for monoacetone rhamnofuranose, except that 5 per cent of absolute methyl alcohol was added to the acetone. The entire product distilled without decomposition at 100–105° under 0.7 mm. pressure. Two fractions were collected. n_D^{26} = 1.4487 (first fraction), n_D^{26} = 1.4520 (second fraction).

The specific rotation of the first fraction was

$$[\alpha]_D^{20} = \frac{-3.09° \times 100}{2 \times 2.315} = -66.7° \text{ (in methyl alcohol)}$$

$$[\alpha]_D^{20} = \frac{-1.86° \times 100}{2 \times 1.980} = -46.9° \text{ (in water)}$$

The specific rotation of the second fraction was

$$[\alpha]_D^{20} = \frac{-1.85° \times 100}{2 \times 1.766} = -52.4° \text{ (in methyl alcohol)}$$

$$[\alpha]_D^{25} = \frac{-1.60° \times 100}{2 \times 2.230} = -35.8° \text{ (in water)}$$

Both fractions had the same composition and consisted of mixtures of α and β forms. The following analysis was obtained from the first fraction.

5.760 mg. substance: 11.650 mg. CO_2 and 4.300 mg. H_2O
3.070 " " : 3.319 " AgI

$C_{10}H_{18}O_5$.	Calculated.	C 55.02, H 8.3, OCH_3 14.22
218.1	Found.	" 55.15, " 8.3, " 14.09

Methylation of Monoacetone Methylrhamnofuranoside—49 gm. of monoacetone methylrhamnofuranoside were methylated according to the method of Muskat.[5]

The potassium salt of monoacetone methylrhamnofuranoside is a white, crystalline substance. It is quite soluble in liquid ammonia and in anhydrous ether.

The salt reacts very violently with methyl iodide. It is therefore advisable to use ether as a diluent, and to add the methyl iodide (slightly in excess of the theoretical quantity) slowly and with constant shaking. The entire reaction was carried out at the temperature of an ice-salt bath. The reaction mixture was shaken for 10 minutes after all of the methyl iodide had been added. In order to insure that the reaction was complete and that all the potassium salt had reacted with the methyl iodide, a sample of the product was removed and dissolved in water. The aqueous solution was neutral, showing that the reaction had reached completion.

The ether was removed by evaporation and the reaction product was extracted several times with chloroform. The ether and chloroform extracts were combined, filtered, and the solvents removed by vaporization. The residual oil distilled completely at 65° under 0.1 mm. pressure. Yield, 48 gm. (92.3 per cent). $n_D^{26} = 1.4360$.

Its specific rotation was

$$[\alpha]_D^{26} = \frac{-4.94° \times 100}{2 \times 4.532} = -54.5° \text{ (in methyl alcohol)}$$

The substance had the same composition as, and is identical with, the monomethyl monoacetone methylrhamnofuranoside prepared by the methylation of monoacetone rhamnofuranose by the Purdie method described above.

Preparation of p-Toluenesulfonyl Monoacetone Methylrhamnofuranoside (V)—*p*-Toluenesulfonyl chloride (1.6 gm.) was added to a solution of acetone methylrhamnofuranoside (1 gm.) in dry pyridine (1.5 cc.). The mixture was allowed to stand overnight at room temperature and then dissolved in chloroform with the addition of water. The aqueous portion was extracted twice with small quantities of chloroform. The combined chloroform extracts were then washed successively with dilute hydrochloric acid, dilute sodium hydroxide, and water. The chloroform solution was now dried over anhydrous sodium sulfate, filtered, and the solvent removed by vaporization under diminished pressure.

The product was a pale yellow syrup which crystallized from methyl alcohol in white needles, m.p. 80°. The compound was very soluble in the usual organic solvents with the exception of ligroin.

Its specific rotation was

$$[\alpha]_D^{24} = \frac{-0.40° \times 100}{1 \times 3.270} = -12.2° \text{ (in } CH_3OH\text{)}$$

The substance had the following composition.

5.101 mg. substance: 10.200 mg. CO_2 and 2.755 mg. H_2O
10.050 " " : 6.505 " $BaSO_4$

$C_{17}H_{24}O_7S$.	Calculated.	C 54.80, H 6.5, S 8.60
372.26	Found.	" 54.52, " 6.1, " 8.95

2 gm. of the *p*-toluenesulfonyl monoacetone methylrhamnofuranoside were heated in a sealed tube for 2 hours with 2 gm. of sodium iodide and 10 cc. of acetone at 100°. The solution was then evaporated to dryness under diminished pressure, and the dry residue extracted repeatedly with hot chloroform. The chloroform extracts were washed with dilute sodium thiosulfate solution and then with water. The chloroform extract was dried and the solvent removed by vaporization.

The resulting oil (2 gm.) was crystallized from methyl alcohol and melted at 80°. A mixture with the original *p*-toluenesulfonyl monoacetone methylrhamnofuranoside also melted at 80°. A qualitative test for halogen was negative. This is in accord with previous observations that a *p*-toluenesulfonyl group attached to a secondary alcoholic group cannot be replaced under these conditions.

Preparation of Monomethyl Rhamnofuranose (IV)—The monomethyl monoacetone methylrhamnofuranoside (14 gm.) was hydrolyzed with 1.5 per cent sulfuric acid (300 cc.) for 90 minutes at the boiling point (according to the method of Freudenberg[4]). All the monomethyl monoacetone methylrhamnofuranoside had dissolved within that time. The solution was rendered neutral with barium carbonate, filtered, and the water removed under diminished pressure, at a temperature not exceeding 40°. The residual syrup was dissolved in acetone and filtered from barium salts. The acetone was removed by vaporization under diminished pressure and a syrup similar to that described by Freudenberg was obtained.

A sample of the syrupy monomethyl rhamnofuranose was oxidized with silver oxide according to the method of Freudenberg.[4] The silver salt of methoxylactic acid was isolated exactly as described by him.

Preparation of Triacetyl-Monomethyl-Rhamnofuranose (VI)—10 gm. of monomethyl rhamnofuranose were dissolved in a solution of 20 cc. of acetic anhydride in 25 cc. of pyridine. The solution was allowed to stand at 0° overnight and then poured onto ice with shaking.

The ice-cold aqueous solution was extracted several times with small quantities of chloroform and the combined chloroform extracts were washed with dilute sulfuric acid, sodium hydroxide, and finally with water. The chloroform solution was then dried over anhydrous sodium sulfate and the solvent removed under diminished pressure.

The residual syrup distilled almost completely at 128–131° under 2 mm. pressure. On standing, it crystallized in part. The crystals were separated and recrystallized from methyl alcohol. They melted at 114°.

The specific rotation of the solid was

$$[\alpha]_D^{25} = \frac{-0.45° \times 100}{2 \times 0.340} = -66.2° \text{ (in methyl alcohol)}$$

The specific rotation of the liquid was

$$[\alpha]_D^{25} = \frac{-0.58° \times 100}{1 \times 2.06} = -28.1° \text{ (in methyl alcohol)}$$

The crystalline form must have the α structure since in the *l* series the difference between the rotatory values of $\alpha - \beta$ should be negative.[6] Both the solid and liquid forms had the same composition. The following analysis was obtained for the crystalline product.

4.585 mg. substance: 8.555 mg. CO_2 and 2.570 mg. H_2O
7.695 " " : 6.065 " AgI
110.8 " " required 11.05 cc. 0.1 N NaOH
$C_{13}H_{20}O_8$. Calculated. C 51.26, H 6.6, OCH_3 10.20, $COCH_3$ 42.43
304.2 Found. " 51.06, " 6.3, " 10.40, " 42.80

A portion of the triacetyl-methyl-rhamnofuranose was deacetylated with barium methylate according to the method of Levene and Tipson.[7] An effort was then made to crystallize the 5-methyl rhamnose, but without success.

Preparation of Bromo-Diacetyl-Monomethyl-Rhamnofuranose (*VII*)—10 gm. of triacetyl-monomethyl-rhamnofuranose were mixed with 50 cc. of glacial acetic acid containing 40 per cent of dry hydrogen bromide.[8] The resulting solution was allowed to stand at room temperature for 60 minutes, after which the hydrogen bromide gas was removed under diminished pressure at room temperature. The solution was then diluted with 100 cc. of toluene and evaporated to a thick syrup under diminished pressure at 35°. Two further portions of 50 cc. of toluene were run in and evaporated off. This syrup was now dissolved in 50 cc. of benzene and the solution was evaporated to a thick syrup. Traces of solvent were removed under a high vacuum at 35°.

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[6] Hudson, C. S., *J. Am. Chem. Soc.*, **31**, 66 (1909).

[7] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **93**, 631 (1931).

The resulting thick, pale yellow syrup was dissolved in dry ether, a little charcoal was added, the mixture shaken and filtered through a fluted filter, the filtrate obtained being faintly yellow. The filtrate was then concentrated by evaporating the ether under suction. The rapid evaporation of the ether cooled the solution and crystallization set in. The crystals were collected on a filter and washed with a mixture of ether and pentane (1:1). The colorless crystals melted at 100.5°.

The substance had the following composition.

8.570 mg. substance: 4.690 mg. AgBr (direct precipitation)

$C_{11}H_{17}O_6Br$.	Calculated.	Br 24.6
325.05	Found.	" 23.3

Its specific rotation was

$$[\alpha]_D^{24} = \frac{-2.15^\circ \times 100}{2 \times 0.610} = -176.2^\circ \text{ (in dry chloroform)}$$

Preparation of Monomethyl-Diacetyl-Theophylline-Rhamnofuranoside (IX)—2 gm. of bromo-diacetyl-monomethyl-rhamnofuranose were dissolved in 100 cc. of dry xylene. 2 gm. of the silver salt of theophylline, dried to constant weight at 140° under reduced pressure, were added and the mixture was kept at about 90° overnight, after which no bromine was found in a small filtered test portion.

The mixture was filtered, and the almost colorless filtrate was shaken with charcoal and filtered again. On standing overnight at room temperature a small amount of theophylline was deposited.

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[8] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **92**, 109 (1931).

The filtrate from this was poured into 2 liters of ligroin and the precipitate collected and dried. It has no definite melting point. It shrinks at about 62° and melted at about 83°. This is charteristic of similar theophylline derivatives.

Its specific rotation is

$$[\alpha]_D^{24} = \frac{-0.55° \times 100}{2 \times 1.184} = -23.2° \text{ (in methyl alcohol)}$$

The substance had the following composition.

5.190 mg. substance: 9.625 mg. CO_2 and 2.730 mg. H_2O
1.940 " " : 0.215 cc. N (757 mm., 29°)
$C_{18}H_{24}O_8N_4$. Calculated. C 50.94, H 5.66, N 13.2
424.1 Found. " 50.60, " 5.88, " 12.5

Preparation of 1,2-Methylorthoacetyl-3-Acetyl-5-Methyl-Rhamnofuranose (VIII)—The orthoacetate was prepared according to the method of Haworth.[9] 1 gm. of the bromo-diacetyl-monomethyl-rhamnofuranose was dissolved in dry methyl alcohol containing 2 cc. of quinoline and the solution allowed to stand in the refrigerator during 3 hours. 50 cc. of chloroform were then added and the quinoline was removed by shaking with dilute sulfuric acid. The chloroform extract was now washed with sodium bicarbonate and finally with water and dried over anhydrous sodium sulfate. The chloroform was removed by evaporation and the residual colorless oil was kept under a high vacuum overnight. $n_D^{24} = 1.4490$.

The specific rotation was

$$[\alpha]_D^{24} = \frac{+0.75° \times 100}{2 \times 1.188} = +31.5° \text{ (in methyl alcohol)}$$

The substance had the following composition.

4.600 mg. substance: 8.800 mg. CO_2 and 2.845 mg. H_2O
5.495 " " : 9.000 " AgI
124.6 " " required 6.75 cc. 0.1 N NaOH
$C_{12}H_{20}O_7$. Calculated. C 52.1, H 7.3, OCH_3 22.4
276.1 Found. " 52.16, " 6.9, " 21.6
Calculated. $COCH_3$ 15.6 for one acetyl group
" 31.2 " two " groups
Found. " 23.2

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[9] Haworth, W. N., *J. Chem. Soc.*, 2862 (1931).

The substance was therefore a mixture of the glycoside and the orthoacetate and no effort was *made to separate them.*

We wish to thank Dr. R. S. Tipson for his kind assistance in the preparation of the manuscript.

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ANALYSIS OF ROTATORY DISPERSION CURVES

II. CONFIGURATIONALLY RELATED SUBSTITUTED FATTY ACIDS

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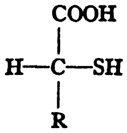
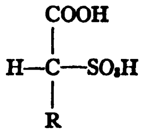
The rotatory dispersion curves of substituted fatty acids of the types



$$\begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{H{-}C{-}SH} \\ | \\ \mathrm{R} \end{array}$$

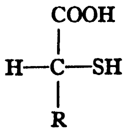
and



$$\begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{H{-}C{-}SO_3H} \\ | \\ \mathrm{R} \end{array}$$

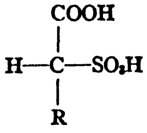
have been measured in the visible and ultraviolet regions. The results are in harmony with the rule previously reported that in members of homologous series, the total rotation of consecutive members may differ in sign, but the signs of the partial rotations remain constant.

The present communication deals with the analysis of the partial rotations of members of two configurationally related homologous series of substances of the following types:



$$\begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{H{-}C{-}SH} \\ | \\ \mathrm{R} \end{array}$$

I.

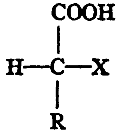


$$\begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{H{-}C{-}SO_3H} \\ | \\ \mathrm{R} \end{array}$$

II.

The members of the two series differ in the character of one substituent, namely, —SH and —SO_3H, respectively. These two groups differ in their chromophoric properties and it was of interest to inquire, first, into the direction of the partial rotations of each of these groups, and second, into the partial rotations of the remaining parts of the molecules which are identical in the members of both series.

A further point of inquiry was the effect of ionization of the carboxyl group on the partial rotations. In carboxylic acids of the type



$$\begin{array}{c} COOH \\ | \\ H—C—X \\ | \\ R \end{array}$$

(where X = any functional group) ionization of the —COOH group was found to produce a marked effect on the value of the rotation of the substance measured in the visible part of the spectrum and frequently to produce a change in the direction of the rotation. It was desired to ascertain whether the ionization of the carboxyl group has a similar rotational effect on the substances of the two series.

Levene, Mori and Mikeska[1] have reported observations on the rotations for the sodium D line of four members of the normal series of 2-mercapto acids and of the four corresponding sulfo acids and certain of their salts. They found that all the members of the series of the mercapto acids rotate in one direction, whereas the rotations of the higher members of the series of sulfo acids were in the opposite sense from those of the lower, the change of sign occurring between the second and third member. On the other hand, the rotations of the salts of the higher mercapto acids were in the opposite direction from those of the lower, but the salts of the sulfo acids all rotated in the same direction.

The following four acids were made the subject of the present investigation.

Dextro 2-mercaptobutyric acid (1) in its *Levo* monosodium salt.
Dextro 2-mercaptocaproic acid (1) and its *Dextro* monosodium and *Levo* disodium salts.
Dextro 2-sulfobutyric acid (1) and its *Levo* disodium and *Levo* calcium salts.
Levo 2-sulfocaproic acid (1) and its *Levo* disodium salt.

Inasmuch as interpretation of the dispersion curves depends on a knowledge of the absorption curves, the latter were made the subject of special study.

The results of the study of the absorption and dispersion curves may be summarized as follows:

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[1] P. A. Levene, T. Mori and L. A. Mikeska, J. Biol. Chem. 75, 337 (1927).

Absorption

Mercapto Acids

The absorption curves of the two mercapto acids studied exhibit a definite maximum at 2400A. This band can safely be attributed to the SH group, inasmuch as 2-mercaptobutane has an absorption band in the same part of the spectrum, which is, however, slightly displaced towards higher frequencies (as is to be expected), and yet is still in a region of transparency for a simple aliphatic acid. The absorption curves of the sodium salts are less characteristic owing to the appearance of a strong second band (COO^-) which overlaps the SH band and masks its maximum (see Fig. 1).

Sulfo Acids

The absorption curves of the sulfo acids present a characteristic maximum at 2150A (see Fig. 2). This band is doubtless the first COOH band displaced towards lower frequencies by the proximity of COOH to SO_3H, since we ascertained that an ordinary alkylsulfonic acid (2-methylpropylsulfonic acid (1)), has practically no absorption at all in that region and absorbs much less than the corresponding aliphatic acid.

The absorption curves of the sodium salts present a striking difference. The curve is displaced towards higher frequencies by about 150A, the maximum of the first band can no longer be seen and a continuous increasing absorption is observed as the wave-length becomes smaller. The fact that we have two bands overlapping each other and not one broad band is very well demonstrated by analysis of both absorption and dispersion curves. The striking change in the absorption is brought about by the ionization of the COOH group since the SO_3H group is already completely ionized in the free acid, as electrometric titration curves have shown.

Rotation

Mercapto Acids

Analysis of the dispersion curves and direct dichroism measurements have shown that the first SH band is strongly coupled and that its rotatory contribution is predominant, i.e., the total molecular rotation has the sign of the SH partial rotation due to anisotropy of the —SH band. The remainder of the rotation, the major part of which can be attributed to the anisotropy of the COOH group, is of opposite sign.

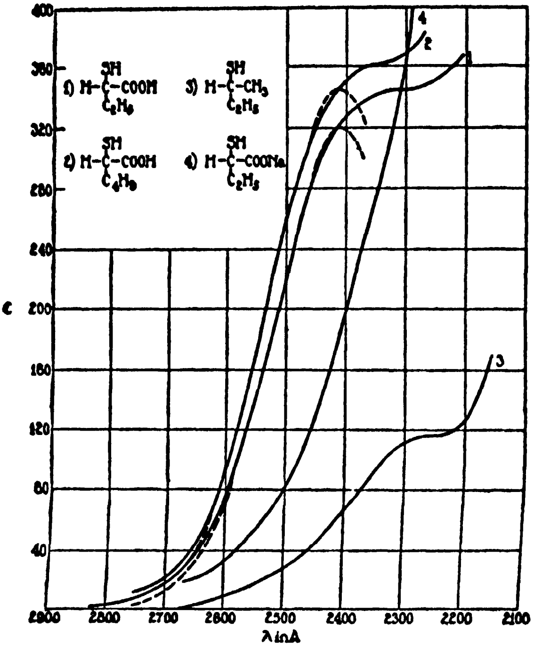


FIG. 1. Absorption curves of the mercapto acids and their sodium salts. The dotted curve is calculated.

The dispersion curves may be expressed by two dispersion terms (of Drude formula) of opposite sign, the dispersion constants λ_1 being practically the same for both compounds and the same being true for the λ_2 (see Tables III and V).

Sulfo Acids

It was stated above that the rotations of the higher members of this series, beginning with the third, are of opposite sign from those of the lower.

The change in sign is due not to the change in sign of the partial rotations of the corresponding anisotropic groups, but to the fact that the absolute magnitude of the second partial contribution is greater than that of the first for the higher members, as a result of which their dispersion is anomalous. (Dispersion curve of case 4, see Levene, Rothen and Marker, reference 2, page 664.) The analysis of the dispersion curves of the free acids shows (see Tables VIII and XI) that the total rotation may be expressed by two terms of opposite sign, the

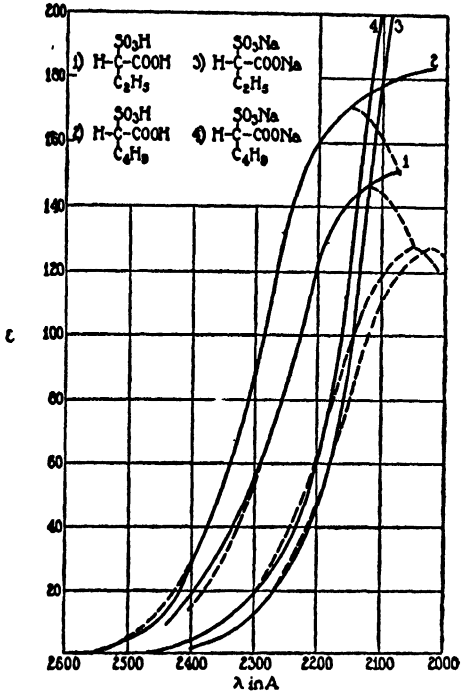
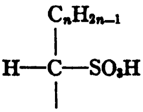


FIG. 2. Absorption curves of the sulfo acids and their sodium salts. The dotted curve is calculated.

first term having a constant of dispersion corresponding to the first absorption band of the carboxyl group (2120A for 2-sulfobutyric acid (1), 2150A for 2-sulfocaproic acid (1)). In the case of 2-sulfobutyric acid the second term is negative and the dispersion constant λ_2 is less than zero, indicating clearly an anomalous course of this term. In the case of 2-sulfocaproic acid the second term is likewise negative and the value of λ_2 approaches zero, thus indicating a similar anomaly.

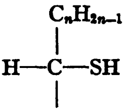
These observations are in harmony with the rule, deduced from previously reported observations, to the effect that in *members of homologous series the total rotations of consecutive members may differ in sign but the signs of the partial rotations remain constant.*[2] Recently, Kuhn[3] made similar observations on the configurationally related inorganic complex salts.

If we consider the sulfo acids in relation to the mercapto acids, we see that the change SH→SO_3H produces a great rotatory effect which in small part may be due to the ionization of the group SO_3H. It may be mentioned, however, that the direction of the acid remains the same in an excess of mineral acid. The anisotropy of the carboxyl group is of opposite sign in the two series. In other words, the vicinal effect of the groups



$$\begin{array}{c} C_nH_{2n-1} \\ | \\ H—C—SO_3H \\ | \end{array}$$

and



$$\begin{array}{c} C_nH_{2n-1} \\ | \\ H—C—SH \\ | \end{array}$$

induces opposite anisotropies in the carboxyl group.

Salts of Mercapto Acids

The opposite rotations observed in the visible region for 2-mercaptobutyric acid (1) and its salt are easily understood, since the dispersion curve of the salt has been found to be anomalous. No change of sign of anisotropy occurs on passing from the acid to the salt, but only a decrease in the magnitude of the partial rotation of the SH group which for the salt becomes smaller than the second contribution for $\lambda > 4046$ 6A. Although the dispersion curves of the monosodium salts of these acids seem distinctly different, they can be expressed with precision by two dispersion terms having the same constants λ_1 and λ_2, respectively (see Tables IV and VI). The change of sign between 2-mercaptocaproic acid (1) and its disodium salt is easily explained in the same way (see Tables V and VII). In short, the anisotropy of the SH band increases from the lower to the higher members of the series and is greater for the free acid than for its salts.

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[2] P. A. Levene, Al. Rothen and R. E. Marker, Chem. Phys. **1**, 662 (1933).

[3] W. Kuhn and K. Bein, Zeits. f. anorg. Chem. **216**, 321 (1934).

Salts of the Sulfo Acids

The striking differences between the absorption of the sulfo acids and their salts are also found in their rotatory behavior. The dispersion curves of the salts can be resolved into two terms of opposite sign, each of them being of the opposite sign to the corresponding term for the free acid (see Tables IX, X and XII). The constant λ_1 of *the first term* is about 100A smaller than the corresponding λ_1 of the first term for the acid. It is very probable that the constants λ_1 of the acid and salt correspond to two different electronic states, despite the fact that they are of almost the same magnitude.

Kuhn[4] arrived at analogous conclusions in a somewhat similar case when interpreting the dispersion curve of octyl nitrite. He assumed two different electronic states in the absorption band of NO_2 to explain two anisotropy factors of opposite sign in the same region of the spectrum.

The *second term* is strongly dispersive (λ_2 $-$1924A) in contrast to the anomalous second term of the acid.

Attention must be called to the fact that the dispersions of the thio and the sulfo acids were measured in different solvents. It is known that the direction of rotation of some substances changes with the change of solvent. However, this change was found to be due to the effect of the solvent on the numerical values of the individual partial rotations, the directions of the latter remaining unaltered.* In the special case of the thio-derivatives, the direction of rotation remains the same for both substances in ether or in water containing enough alcohol to effect the solution of the thio-derivatives.

Shape of Absorption Curves

The absorption curves of the mercapto acids, the sulfo acids and their salts were analyzed and it was found that they could be expressed by an exponential equation based on a Maxwellian distribution (as was first done by V. Henri,[5] and since then by Kuhn,[6] Lowry[7] and others.[7] In accord with the recent work of Lowry,[4] we found that better agreement between experimental and calculated rotation values was obtained by using a wave-length scale instead of a frequency scale. Consequently, our absorption curves have been expressed in the wave-length scale. We give below the parameters of the absorption curves for the different compounds, as calculated from the equation

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[4] W. Kuhn and H. L. Lehmann, Zeits. f. physik. Chemie **B18**, 32 (1932).

* See W. Kuhn, *Stereochemie* of K. Freudenberg, p. 416, 1933 and Levene-Rothen-Meyer, Science **80**, 101 (1934).

$$\epsilon = \epsilon_{\text{max.}} e^{-[(\lambda-\lambda_0)/\theta]^2}$$

(where $\theta = \lambda'/1.665$, λ' is the half-width of the band and ϵ is the molec-

TABLE I

Absorption Constants

Compound	Solvent	$\epsilon_{\text{max.}}$	λ_0	θ
2-Mercaptobutyric acid (1)	heptane	320	0.2410	0.0159
2-Mercaptocaproic acid (1)	heptane	345	0.2410	0.0172
2-Sulfobutyric acid (1)	water	147	0.2120	0.0172
Na salt of 2-sulfobutyric acid (1)	water	128	0.2025	0.0180
2-Sulfocaproic acid (1)	water	172	0.2150	0.0180
Na salt of 2-sulfocaproic acid (1)	water	128	0.2050	0.0180

TABLE II

*Circular Dichroism**

Compound	λ in A	$(\epsilon_l - \epsilon_r)\lambda$	$(\epsilon_l - \epsilon_r)\lambda_{2410}$ Calc.	ϕ Calc. from dichroism data	ϕ Calc. from dispersion and absorption data
2-Mercaptobutyric acid (1)	2480	1.46 ±0.15	1.8_5	3700°	3600°
2-Mercaptocaproic acid (1)	2450	2.10 ±0.30	2.3	4600°	4680°
	2560	0.80 ±0.08			

* See the relations between circular dichroism, anistropy factor and maximum rotation of an active band, Hand- und Jahrbuch der chemischen Physik, *Drehung der Polarisationsebene des Lichtes*, von W. Kuhn und K. Freudenberg. Leipzig, 1932, p. 79.

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[5] V. Henri and J. Bielecki, Phys. Zeits. **14**, 516 (1913).

[6] W. Kuhn and E. Braun, Zeits. f. physik. Chemie **B8**, 281 (1930).

[7] T. M. Lowry and H. Hudson, Phil. Trans. Roy. Soc. **A232**, 117 (1933). H. Hudson, M. L. Wolfrom and T. M. Lowry, J. Chem. Soc. 1933, 1179.

ular extinction coefficient as defined in the *International Critical Tables*). Wave-lengths are expressed in microns to comply with the usual convention adopted in polarimetry. As may be seen from Figs. 1 and 2, only that part of the curve on the longer wave-length side of the origin λ_0 could be observed (see Table I).

It is interesting to note that in the case of the sodium salts of the two sulfo acids for which no maximum could be detected, the λ_0 which had to be chosen in order to represent correctly the first part of the absorption curve, had the same value as that found by dispersion measurements.

Rotatory Dispersion Formulae

According to Drude, the equation representing the rotation in the neighborhood of an active absorption band, has the form

$$[M] = \frac{A(\lambda^2 - \lambda_0^2)}{(\lambda^2 - \lambda_0^2)^2 + \lambda'^2\lambda^2}.$$

The dispersion calculated from a simple term $A/(\lambda^2 - \lambda_0^2)$ fixes the upper limit of the rotation. The rotation as we approach λ_ϕ (ϕ = maximum rotation) increases, although it never reaches the value calculated from a simple term. On the other hand, the main characteristic of Kuhn's[6] recent dispersion formula (written with a λ scale as used by Lowry[7])

$$[M] = \frac{\phi}{m}\frac{\lambda_\theta}{\lambda}\left[e^{-[(\lambda-\lambda_0)/\theta]^2}\int_0^{(\lambda-\lambda_0)/\theta} e^{x}dx + \frac{\theta}{2(\lambda+\lambda_0)}\right],$$

where *m* is the maximum value of the term inside the bracket, is that, for values of λ between $\lambda_{0+4\theta}$ and λ_ϕ the rotation increases more rapidly than if it were following a simple Drude term. Consequently, if a simple Drude term is used to express a dispersion over a wave-length interval which goes beyond the critical value $\lambda_{0+4\theta}$ then the value found for λ_0 is greater than the true value. We have found that, whenever sufficient precision was attained in the rotatory measurements in the neighborhood of the absorption band, Kuhn's equation was more satisfactory than Drude's. This is very well illustrated in Tables III and V. The figures in columns 4 are calculated from a formula having two Drude terms, the constant λ_1 of the first term being

TABLE III

Rotatory Dispersion of Dextro-2-Mercaptobutyric Acid (1) in Heptane

Visible region: Concentration 1.146_7M, $l = 20$ cm. U.V. region: Concentration *1.146_7 from 3990A to 3005A, 0.1147 from 2980 to 2770, 1 = 5 cm from 3990A to 2843A, 1 cm from 2768A to 2370A. Measurements on levo form.* The figures in the last column ($[M]^{25}_{\text{calc.}}$) are calculated from the equation

$$[M]^{25}\text{ calc.} = \frac{\phi}{0.556}\frac{\lambda_\phi}{\lambda}\left[e^{-[(\lambda-\lambda_0)/\theta]^2}\int_0^{(\lambda-\lambda_0)/\theta} e^{x^2}\,dx + \frac{\theta}{2(\lambda+\lambda_0)}\right] - \frac{19.578}{\lambda^2 - 0.043}.$$

$$\phi = 3600^\circ, \qquad \lambda_0 = 0.245, \qquad \theta = 0.01537, \qquad \lambda_\phi = 0.2560.$$

This equation reduces for $\lambda > 0.3065$ to

$$[M]^{25}_{\text{calc.}} = \frac{25.495_5}{\lambda^2 - 0.060} - \frac{19.578_4}{\lambda^2 - 0.043}.$$

λ in A	α^{25}	$[M]^{25}$	$[M]^{25} = \frac{19.1614}{\lambda^2 - 0.064} - \frac{13.2006}{\lambda^2 - 0.042}$	$[M]^{25}_{\text{calc.}}$
5892.6	5.605	24.440	24.40	24.41
5780.1	5.907_5	25.759	25.75	25.76
5460.7	6.947	30.293	30.29	30.31
4358.3	14.44	62.96	62.91	62.96
4046.6	19.19	83.67_5	83.67	83.59
3990	5.10	88.9_5	88.64	88.52
3815	6.10	$106._4$	107.5	107.1
3690	7.10	$123._8$	125.3	124.6
3593	8.10	$141._3$	142.8	141.6
3518	9.10	$158._7$	159.2	157.4
3445	10.10	$176._1$	178.3	175.8
3340	12.20	$211._0$	213.2	208.9
3260	14.10	$245._9$	247.9	241.8
3197	16.10	$280._8$	282.3	273.3
3145	18.10	$315._7$	317.0	
3098	20.10	$350._6$	354.6	339.1
3005	26.10	$455._2$	455.2	
2980	2.80	488	490.5	
2950	3.10	541	539.0	
2908	3.60	628	621.7	630.
2870	4.10	715	716.2	740.
2855	4.35	759	760	
2843	4.60	802	799	825
2770	1.27	1102	1125	1215
	conc. 0.0115M			
2620	0.30	2600		2660
2560	0.30	2600		2730
2483				430
2370	−0.29	−5100		−4650
	conc. 0.057M			

80A greater than the real value. Experimental and calculated rotations are in very good agreement over a considerable wave-length interval extending far beyond the critical value $\lambda_{1+4\theta}$ but, for values of λ approaching λ_{ϕ}, the calculated values have no longer any significance. For the near ultraviolet region, the figures found in columns 5 (which are calculated from a two term formula the first of which is a Kuhn term) do not check with the experimental values as well as do the figures in column 4, but are quite satisfactory for values of λ well within the band. Furthermore, the value of λ_1 of the Kuhn term is very near that found from absorption measurements for the head of this particular band. Part of the dispersion curve of the sodium salt of 2-sulfocaproic acid (1) may be seen in Fig. 3 (see Table XII). We have chosen this particular example since it illustrates very clearly how two different causes can produce an apparent shift of the value of

TABLE IV

Rotatory Dispersion of Levo Monosodium Salt of 2-Mercaptobutyric Acid (1) in Water Containing 25 Percent CH_3OH

Concentration: 0.7043M

Visible region: l = 20 cm

U.V. region: l = 5 cm from 3500 to 3110, 0.5 cm from 2950 to 2780. Measurements on dextro form.

λ in A	α^{25}	$[M]^{25}$	$[M]^{25} = \frac{5.150}{\lambda^2 - 0.062} - \frac{6.264}{\lambda^2 - 0.040}$
5780.1	−0.334	−2.37	−2.37
5460.7	−0.350	-2.48_5	−2.46
4358.3	−0.225	−1.60	−1.52
4046.6	0.00	0.00	0.00
3740	+0.15	+4.3	+3.45
3610	+0.25	+7.1	+6.0
3560	+0.35	+9.9	+7.3
3330	+0.65	$+18._5$	+16.9
3245	+0.85	$+24._1$	+23.1
3180	+1.05	$+29._8$	+29.2
3110	+1.25	$+35._5$	+37.9
2950	+0.25	+71	+72.6
2900	+0.35	+99	+91.0
2850	+0.45	+128	+116.0
2790	+0.55	+156	+159.6

TABLE V

Rotatory Dispersion of Dextro-2-Mercaptocaproic Acid (1) in Heptane

Visible region: concentration 0.6855M, l = 20 cm.

U.V. region: concentration 0.6855M from 3965A to 2703A, 0.2285M for 2665A, 0.114M from 2585A to 2550A, 0.0571M from 2380A to 2340A, l = 5 cm from 3965A to 2980A, 0.5 cm from 2920 A to 2825A, 0.1 cm from 2785A to 2340A.

The figures in column 5 are calculated from

$$[M]^{25}_{\text{calc.}} = \frac{\phi}{0.554}\,\frac{\lambda_\phi}{\lambda}\left[e^{-[(\lambda-\lambda_0)/\theta]^2}\int_0^{(\lambda-\lambda_0)/\theta} e^{x^2}\,dx + \frac{\theta}{2(\lambda+\lambda_0)}\right] - \frac{20.140}{\lambda^2 - 0.045},$$

where $\phi = 4680°$, $\lambda_0 = 0.245$, $\theta = 0.0132$, $\lambda_\phi = 0.255$.

This equation reduces for $\lambda > 0.3000$ to

$$[M]^{25}_{\text{calc.}} = \frac{28.415}{\lambda^2 - 0.060} - \frac{20.140}{\lambda^2 - 0.045}.$$

λ in A	α^{25}	$[M]^{25}$	$[M]^{25} = \frac{20.249}{\lambda^2 - 0.064} - \frac{11.973}{\lambda^2 - 0.042}$	$[M]^{25}_{\text{calc.}}$
5780.1	4.662	34.00_4	33.98	34.00
5460.7	5.447	39.73	39.73	39.75
4358.3	10.93	79.72	79.85	79.72
4046.6	14.34	104.5_9	104.3	104.3
3965	3.90	$113._8$	113.3	112.8
3750	4.90	$143._0$		
3590	5.90	$172._1$	174.3	172.4
3482	6.85	$199._8$		
3393	7.85	$229._0$	232.4	228.3
3322	8.85	$258._2$		
3215	10.85	$316._6$	319.3	310.2
3170	11.85	$345._7$	350.3	
3100	13.85	$404._1$		
3070	14.85	$433._2$	440.3	420.7
3042_5	15.85	$462._4$	472.0	449.1
3020	16.85	$491._6$	501.1	
2998	17.85	$520._8$		474.8
2980	18.85	$550._0$	560.7	526.8
2920	2.30	671	675.7	
2868	2.80	817	812.1	803
2825	3.30	948	964.4	965
2785	0.80	1170	1157	1173
2760	0.90	1310	1302	1330
2703	1.30	1900	1850	1940
2665	0.60	2620	2470	2500
2585	0.40	3510	6698	3650
2550	0.43	3800		3670
2380	−0.28	−4900		−5800
2340	−0.40	−7000		−6900

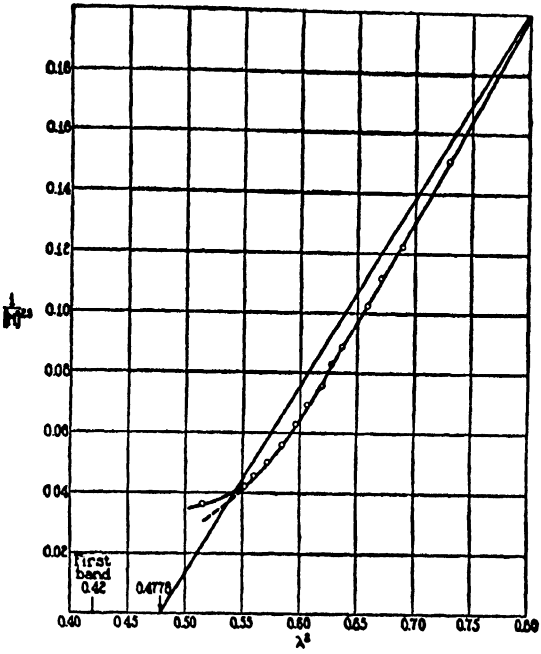

FIG. 3. Dispersion curve of the sodium salt of sulfocaproic acid. The dotted curve is calculated.

TABLE VI

Rotatory Dispersion of Dextro Monosodium Salt of 2-Mercaptocaproic Acid (1) in Water Containing 30 Percent CH_3OH

Concentration: 0.3106M
Visible region: l = 20 cm
U.V. region: l = 5 cm

λ in A	α^{25}	$[M]^{25}$	$[M]^{25} = \frac{5.767}{\lambda^2 - 0.062} - \frac{3.848}{\lambda^2 - 0.040}$
5780.1	+0.504	+8.11	+8.11
5460.7	+0.587	+9.45	+9.51
4358.3	+1.21	+19.5	+19.4
4046.6	+1.59	+25.6	+25.6
3700	+0.60	+38.$_6$	+37.2
3450	+0.85	+54.$_7$	+52.5
3330	+1.10	+70.$_8$	+63.7

TABLE VII

Rotatory Dispersion of Levo Disodium Salt of 2-Mercaptocaproic Acid (1) in Water Containing 20 Percent CH_3OH

Concentration: 0.236M
Visible region: $l = 20$ cm
U.V. region: $l = 5$ cm

λ in A	α^{25}	$[M]^{25}$
5780.1	−0.244	−5.17
5460.7	−0.269	−5.70
4358.3	−0.255	-5.40_5
4046.6	−0.15	−3.18
3480	+0.35	+30
3350	+0.60	+51

TABLE VIII

Rotatory Dispersion of Dextro 2-Sulfobutyric Acid (1) in Water

Concentration: 0.8630M
Visible region: $l = 40$ cm
U.V. region: $l = 5$ cm from 3670A to 2835A, $l = 1$ cm from 2620A to 2535A
Measurements on levo form.

λ in A	α^{25}	$[M]^{25}$	$[M]^{25} = \frac{3.0656}{\lambda^2 - 0.045} - \frac{2.1733}{\lambda^2 + 0.030}$
5875.6	1.535	4.446	4.419
5780.1	1.600	4.635	4.635
5460.7	1.894	5.486	5.486
4358.3	3.89	11.27	11.27
4046.6	5.04	14.60	14.60
3670	0.90	20.8	21.0
3580	1.00	23.2	23.1
3500	1.10	25.5	25.3
3280	1.45	33.6	33.2
3160	1.70	39.4	39.1
3050	2.00	46.3	46.2
2975	2.25	52.1	52.1
2900	2.55	59.1	59.3
2835	2.80	64.9	67.0
2620	0.90	104	107.6
2585	1.00	116	118.0
2535	1.10	127	136.1

TABLE IX

Rotatory Dispersion of Levo Disodium Salt of 2-Sulfobutyric Acid (1) in Water

Concentration: 0.6517M.

Visible region: $l = 40$ cm

U.V. region: $l = 5$ cm from 3450 to 2522A, 0.5 cm from 2500 to 2370A, 0.1 cm for (2335 and) 2320A.

Measurements on dextro form. The figures in the last column are calculated from the equation

$$[M]^{25}_{\text{calc.}} = -\frac{\phi}{0.562}\frac{\lambda_\phi}{\lambda}\left[e^{-[(\lambda-\lambda_0)/\theta]^2}\int_0^{(\lambda-\lambda_0)/\theta} e^{x^2}dx + \frac{\theta}{2(\lambda+\lambda_0)}\right] + \frac{28.640}{\lambda^2 - 0.037}.$$

$$\phi = 5140°, \qquad \lambda_\phi = 0.219, \qquad \lambda_0 = 0.2025, \qquad \theta = 0.0180.$$

for $\lambda > 0.2750$ this equation reduces to

$$[M]^{25} = -\frac{36.039_5}{\lambda^2 - 0.041} + \frac{28.640}{\lambda^2 - 0.037}.$$

λ in A	α^{25}	$[M]^{25}$	$[M]^{25} = -\frac{7.4323}{\lambda^2 - 0.05385}$	$[M]^{25}_{\text{calc.}}$
5892.6	−6.605	−25.337	−25.333	−25.369
5780.1	-6.923_5	−26.560	−26.560	−26.560
5460.7	−7.947	−30.486	−30.417	−30.486
4358.3	−14.26	−54.70	−54.61	−54.70
4046.6	−17.63	−67.63	−67.63	−67.64
3780	−2.70	−82.9	−83.5	−83.2
3680	−2.95	−90.5	−91.1	−90.8
3585	−3.20	−98.2	−99.5	−98.8
3510	−3.45	−105.9	−107.2	−106.2
3445	−3.70	−113.5	−114.6	−113.2
3330	−4.20	−128.9	−130.3	−128.0
3235	−4.70	−144.2	−146.3	−142.9
3155	−5.20	−159.6	−162.7	−157.8
2840	−8.80	−270.1	−277.7	−253.0
2745	−10.80	−331.4	−345.7	−302.4
2680	−12.80	−392.8	−413.5	
2620	−15.60	−478.7		
2595	−16.80	−515.0		−492
2540	−19.80	−607.6		−598
2522	−20.70	−635.3		−644
2500	−2.30	−705.8		−706
2480	−2.55	−782.6		
2455	−2.80	−859		−862
2418	−3.30	−1013		−1038
2398	−3.80	−1166		
2370	−4.40	−1350		−1384
2320	−1.00	−1535		−1834

TABLE X

Rotatory Dispersion of Levo Calcium Salt of 2-Sulfobutyric Acid (1) in Water

Concentration: 0.33076M, visible region l = 40 cm, U.V. region l = 10 cm from 3980 to 2520A, 5 cm for 2340 and 2320A, 0.1 cm for 2270 and 2250A.

Measurements on dextro form.

The figures in the last column are calculated from the equation

$$[M]^{25} = -\frac{\phi}{0.562}\,\frac{\lambda_\phi}{\lambda}\left[e^{-[(\lambda-\lambda_0)/\theta]}\int_0^{(\lambda-\lambda_0)/\theta} e^{x^2}\,dx + \frac{\theta}{2(\lambda+\lambda_0)}\right] + \frac{32.355}{\lambda^2-0.037}.$$

$\phi = 5760°$, $\lambda_\phi = 0.2190$, $\lambda_0 = 0.2025$, $\theta = 0.0180$.

for $\lambda > 0.2750$ this equation reduces to

$$[M]^{25} = -\frac{40.346}{\lambda^2-0.041} + \frac{32.355}{\lambda^2-0.037}$$

λ in A	α^{25}	$[M]^{25}$	$[M]^{25} = -\frac{8.0476}{\lambda^2-0.0542}$	$[M]^{25}_{\text{calc.}}$
5780.1	−3.804	−28.75	−28.75	−28.75
5460.7	−4.367	−33.01	−32.98	−33.36
4358.3	−7.85	-59.3_8	−59.29	−59.33
4046.6	−9.72	-73.4_7	−73.47	−73.42
3980	−2.55	−77.1	−77.2	−77.2
3670	−3.30	−99.8	−100.0	−99.4
3540	−3.80	−114.9	−113.2	−112.1
3410	−4.30	−130.0	−129.7	−127.8
3300	−4.80	−145.1	−147.1	−138.4
3220	−5.30	−160.2	−162.7	−158.4
3155	−5.80	−175.3	−177.5	−171.9
3045	−6.80	−205.6	−209.0	−199.4
2965	−7.80	−235.8	−238.8	−224.6
2896	−8.80	−266.0	−271.3	−250.8
2830	−9.80	−296.3	−311.0	−281.2
2753	−11.80	−356.7	−372.9	
2688	−13.80	−417.2	−446.1	
2600	−17.80	−538.1		−534
2565	−19.80	−598.6		−611
2540	−21.80	−659.1		−670
2520	−23.80	−719.5		−723
2340	−2.80	−1690		−1800
2320	−3.30	−1995		−2200
2270	−0.70	−2120		−2600
2250	−0.80	−2420		−2775

λ_1 towards lower frequencies. The rotation from 5892.6A to 2822A of this salt, as well as that of the corresponding sulfobutyric acid, may be expressed within less than 0.5 per cent by one Drude term. The value of the apparent λ_1 is 2186A, which is 136A higher than the value found from absorption measurements. This shift is due to our having neglected a second term of opposite sign. Now, for values of λ smaller than the critical value $\lambda_{0+4\theta}$ the dispersion becomes still greater and λ_1 seems shifted still further towards lower frequencies. This second shift is produced because the rotation increases according to a Kuhn term and not a Drude term. In fact, the dispersion curve may be fairly well represented by two terms of opposite sign, the first one being a Kuhn term with a value λ_1 equal to that found from absorption measurements.

TABLE XI

Rotatory Dispersion of Levo 2-Sulfocaproic Acid (1) in Water

Concentration: 0.6830M, visible region l = 40 cm, U.V. region l = 10 cm for 3560 and 3270A, 5 cm, for 3450A, 1 cm for 2700A, 0.1 cm for 2400A.

λ in A	α^{25}	$[M]^{25}$	$[M]^{25} = \frac{3.0626}{\lambda^2-0.0462} - \frac{6.5309}{\lambda^2-0.007}$
5892.6	−2.465	−9.023	−9.022
5780.1	−2.548	−9.326	−9.328
5460.7	−2.809	−10.282	−10.275
4358.3	−3.93	−14.38	−14.39
4046.6	−4.26	−15.59	−15.61
3560	−1.10	−16.1	−16.5
3450	−0.53	−15.5	−16.2
3270	−1.00	−14.6	−14.9
2700	+0.20	+2.9	+15.6
2400	+0.22	+32	+102.2

Circular Dichroism

In the case of the mercapto acids, measurements of circular dichroism were made by the method used by Kuhn.[8] Because of experimental difficulties, our results are not very accurate. However, the order of magnitude of the circular dichroism found for the SH band

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[8] W. Kuhn and E. Braun, Zeits. f. physik. Chemie **B8**, 445 (1930).

TABLE XII

Rotatory Dispersion of Levo Disodium Salt of 2-Sulfocaproic Acid (1) in Water

Concentration: 0.2572M, visible region $l = 40$ cm, U.V. region $l = 10$ cm from 3790 to 2520A, 1 cm from 2520 to 2350A, 0.1 cm for 2270.

The figures in the last column are calculated from the relation.

$$[M]^{25} = -\frac{\phi}{0.562}\frac{\lambda_\phi}{\lambda}\left[e^{-[(\lambda-\lambda_0)/\theta]^2}\int_0^{(\lambda-\lambda_0)/\theta} e^{x^2}dx + \frac{\theta}{2(\lambda+\lambda_0)}\right] + \frac{21.271}{\lambda^2-0.037}.$$

$$\phi = 5290°, \qquad \lambda_\phi = 0.2210, \qquad \lambda_0 = 0.2050, \qquad \theta = 0.0180.$$

for $\lambda > 0.2770$ this expression reduces to

$$[M]^{25} = -\frac{37.437}{\lambda^2 - 0.042} + \frac{21.271}{\lambda^2 - 0.037}$$

λ in A	α^{25}	$[M]^{25}$	$[M]^{25} = -\frac{16.197}{\lambda^2 - 0.04778}$	$[M]^{25}_{\text{calc.}}$
5892.6	−5.560	−54.04	−54.09	−54.09
5780.1	−5.820	−56.57	−56.57	−56.57
5460.7	−6.649	−64.63	−64.68	−64.69
4358.3	−11.725	−113.97	−113.93	−113.97
4046.6	−14.37	−139.7	−139.7	−139.7
3790	−4.35	−169.1	−169.0	−168.8
3720	−4.60	−178.8	−178.8	−178.6
3600	−5.10	−198.3	−198.0	−197.7
3490	−5.60	−217.7	−218.8	−218.3
3410	−6.10	−237.2	−236.4	−235.6
3327	−6.60	−256.6	−257.5	−256.4
3265	−7.10	−276.0	−275.4	−273.9
3150	−8.10	−314.9	−314.8	−312.5
3055	−9.10	−353.8	−355.6	−352.7
2980	−10.10	−392.7	−394.8	−389.2
2920	−11.10	−431.6	−432.1	−424.6
2868	−12.10	−470.4	−469.8	−460.0
2822	−13.10	−509.3	−508.4	−495.8
2761	−15.10	−587.1	−569.3	
2702	−17.10	−664.8	−642.0	
2625	−21.10	−820.4	−766.7	−805
2590	−23.10	−898.1		
2567	−25.10	−975.9		−940
2541	−27.10	−1053.6		
2520	−29.10	−1131.4		
2520	−2.90	−1128		−1110
2504	−3.10	−1205		
2487	−3.40	−1322		
2460	−3.70	−1438		−1395
2445	−4.10	−1594		
2415	−4.60	−1788		−1720
2390	−5.10	−1983		1954
2365	−5.60	−2177		
2350	−6.10	−2372		2390
2270	−0.70	−2720		3330

is quite in accord with the maximum partial rotation contributed by that band as calculated from the dispersion curves. Our results are summarized in Table II.

EXPERIMENTAL

Compounds

The compounds were obtained by the methods previously described by Levene, Mori and Mikeska,[1] with the exception of 2-sulfobutyric acid (1) which was prepared and resolved according to Backer.**

All the compounds were carefully analyzed. Titrations indicated that the mercapto acids were at least 99.8 percent pure. The analyses of the sulfo acids were performed on their Ba salts.***

Apparatus

The instruments described in a previous article[2] were used to determine the rotatory dispersions in the visible and the ultraviolet regions.

** The values of the molecular rotations given are not to be taken as maximum rotations since no attempt was made to resolve the compounds to the maximum, hence no special precaution was taken to determine precisely the *absolute* rotations, as the main purpose of this work was to obtain accurate dispersion measurements. However, the *ratio* of the rotations of the acids to those of their respective salts must be considered as characteristic of the compounds, since the same source of material was used to determine the dispersion of the acids and their salts. Backer and Boer [H. J. Backer, J. H. de Boer, Rec. trav. chim. **43**, 297 (1924)] had previously resolved 2-sulfobutyric acid (1). The value they obtained for the maximum rotation of the calcium salt was $[M]_{5892} = -25.3°$, as compared to our own value $[M]^{25}_{5892} = -27.46°$. (Their value has been recalculated from their own function $[M] = f(c)$ for the concentration at which we made our measurements.) The dispersion data given by these authors for a short wave-length interval are in accord with our own measurements. We determined accurately the following ratio

$$\frac{[M]^{25}_{57801}\text{ Ba salt}}{[M]^{25}_{57801}\text{ acid}} = -6.28°\,,$$

which is not in accord with the value −3.27° recorded by Backer. Furthermore, the dispersion of this acid as given by Backer is completely different from our own curve. These discrepancies induced us to repeat our measurements but we found that our results were readily duplicated.

*** We are indebted to Dr. G. M. Meyer of this department for the preparation of a sample of 2-mercaptobutyric acid (1).

The same general procedure was followed and the same precision is claimed. Absorption measurements were carried out with a Hilger sector photometer in conjunction with a Hilger quartz spectrograph model E316.

Unless otherwise indicated, wave-lengths are expressed in microns in all formulae employed.

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XYLOSEPHOSPHORIC ACIDS. II

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In a previous communication[1] we reported the fact that on phosphorylation of a product which was believed to be 5-benzoyl monoacetone xylose, the only product isolated after hydrolysis was 5-xylose phosphate. Moreover, methylation of this same benzoyl derivative led to a mixture of 3-methyl and 5-methyl xylose.[2] Experiments were cited which indicated the benzoyl compound to be homogeneous,[3] thus suggesting a migration either of the benzoyl group prior to phosphorylation, or of the phosphoryl group during removal of the acetone and benzoyl groups. The problem therefore became that of establishing, if possible, the stage at which migration occurred.

For this purpose it was decided first to confirm the essential homogeneity of the benzoyl monoacetone xylose, and second to make renewed attempts to prepare derivatives of xylose substituted in position (3), with isolation, if possible, of the intermediates and determination of their structure.

In order to confirm the belief that the 5-benzoyl monoacetone xylose was homogeneous, a once crystallized preparation was tolylsulfonated and the product was carefully fractionated. Some unchanged starting material was recovered and, after deducting this, the yield of pure 3-tolylsulfo 5-benzoyl monoacetone xylose was 86 per cent of the theoretical. Of the remaining 14 per cent, about 8 per cent was accounted for by impure fractions of unchanged starting material and of chlorine-containing syrup. No evidence was obtained for the presence of the isomeric 5-tolylsulfo 3-benzoyl monoacetone xylose in any fraction. On this basis of this evidence it is warranted to assume that the benzoyl monoacetone xylose is practically homogeneous.

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[1] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **102**, 347 (1933); the first paper of this series.

[2] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **102**, 331 (1933).

[3] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **102**, 317 (1933).

For the second objective stated above, the preparation of derivatives of xylose substituted in position (3), recourse was had to the 5-benzylcarboxy monoacetone xylose previously described. To correlate definitely the position of the benzoyl group and the benzylcarboxy group in these derivatives, the tolylsulfo benzylcarboxy monoacetone xylose was prepared, the benzylcarboxy group was removed catalytically, and the product benzoylated. The resultant tolylsulfo benzoyl monoacetone xylose was identical with that prepared by tolylsulfonation of the benzoyl monoacetone xylose. Inasmuch as only the mildest conditions were employed, the possibility of rearrangements during these reactions would seem to be minimal.

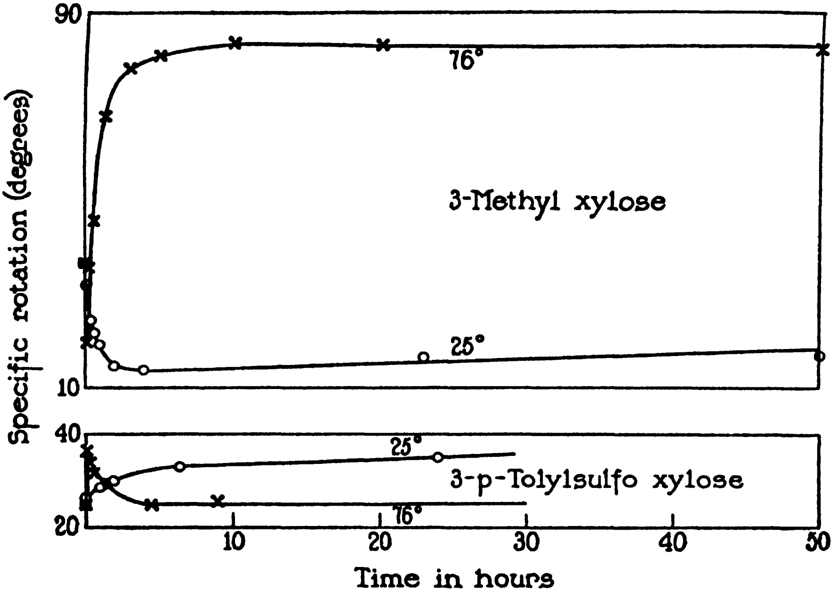


FIG. 1. Glycoside formation on 3-methyl and 3-p-tolylsulfo xylose

The benzylcarboxy tolylsulfo monoacetone xylose was next hydrolyzed to remove both the benzylcarboxy and the acetone groups, and a crystalline tolylsulfo xylose was secured. In experiments on glycoside formation two glycosides, typical in character, were observed, confirming the allocation of the tolylsulfo group to position (3). It is interesting to note (Fig. 1) that although the form of the curves is identical, both the direction and the magnitude of the changes are different in 3-tolylsulfo and in 3-methyl xylose.

Here, therefore, we have a case in which the only product isolated is the 3-substituted xylose, which would be normally expected. It was hoped that 3-phospho xylose might be prepared analogously, and the 5-benzylcarboxy monoacetone xylose was therefore phosphorylated, the acetone removed by acid hydrolysis, and the benzylcarboxy group by catalytic hydrogenation. The isolated phospho xylose was again apparently that with the substituent in position (5).

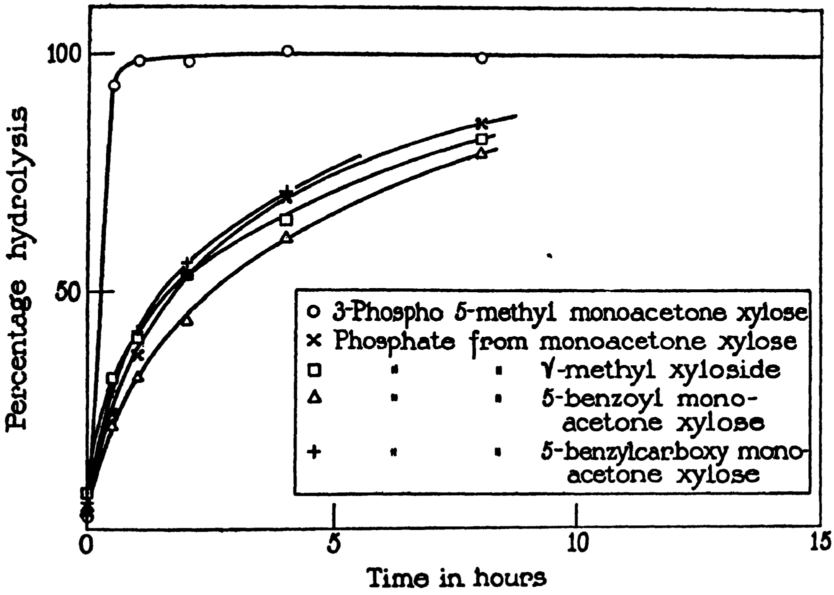
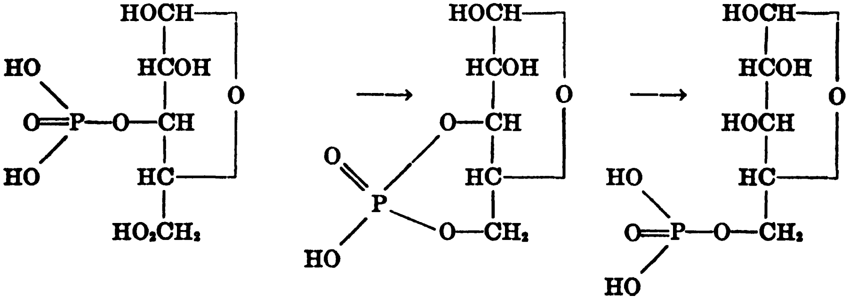


FIG. 2. Rate of phosphorus hydrolysis in phospho xyloses

Because of the small optical changes observed during glycoside formation of the 3-tolylsulfo xylose, it was considered possible that the phospho xyloses described on the basis of glycoside formation as 5-phospho derivatives were really substituted in position (3) and that the optical changes were so small as to be overlooked. The rate of acid hydrolysis of the various phospho xyloses was therefore compared, the inorganic phosphate being determined colorimetrically. The results are given in Fig. 2, from which it may be seen that the 3-phospho 5-methyl monoacetone xylose (in which the phosphoric acid is certainly attached to carbon (3)) has a rate of hydrolysis entirely different from that of any of the other esters. Thus it is evident that the migration of the phosphoryl group occurs in the case of xylose with great ease. This peculiarity may be attributed to the fact that in the case of xylose the hydroxyl groups in positions (3) and (5) are located in close proximity, a fact revealed by the study of the structural model as already pointed out by Haworth.[4] It seems not unlikely that such changes occur through a bridge of the ortho type, as indicated below, although no experimental evidence has been secured for this assumption.



It may be mentioned that an attempt was made to prepare the 3-phospho xylose through the trityl monoacetone xylose but that it was unsuccessful. A further attempt will be made to prepare the compound with 5-benzyl monoacetone xylose as an intermediate.

EXPERIMENTAL

Tolylsulfonation of 5-Benzoyl Xylose—50 gm. of crystalline, but not recrystallized, 5-benzoyl xylose were dissolved in 150 cc. of dry pyridine and 36 gm. of *p*-toluenesulfo chloride were added.

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[4] Haworth, W. N., The constitution of sugars, London, 51 (1929).

The mixture was shaken to dissolve the chloride and was then left overnight at room temperature. 5 cc. of water were added, and after 30 minutes at room temperature the product was isolated in the manner previously described. The dried chloroform solution was concentrated under reduced pressure to a syrup which was dissolved in ether and cooled. Pentane was added to turbidity, and the mixture was seeded. The material obtained was recrystallized similarly, the mother liquors from the two crystallizations being combined. Reworking these mother liquors afforded three additional crops of crystals and a residue which was dried under reduced pressure to a viscous syrup.

Repeated crystallization of the combined fractions from various solvents finally gave 59.7 gm. of pure 3-tolylsulfo 5-benzoyl monoacetone xylose (m.p. 94–95°; $[\alpha]_D^{20} = -64.4°$ to $-64.8°$ ($CHCl_3$)); 3.0 gm. of pure unchanged starting material (m.p. 83–84°; $[\alpha]_D^{20} = +5.5°$ ($CHCl_3$)); 2.9 gm. of impure starting material ($[\alpha]_D^{20} = -6.2°$ ($CHCl_3$)); and 3.6 gm. of a syrup which was found by analysis to contain both sulfur and chlorine. In addition, 1.2 gm. of product were lost during the manipulations. Assuming the impure 5-benzoyl monoacetone xylose recovered above to be 80 per cent pure, the yield of 3-tolylsulfo 5-benzoyl monoacetone xylose is 86 per cent of the theoretical, with 5.4 gm. of impure material towards the remaining 14 per cent. No evidence was secured for the presence of any of the isomeric 3-benzoyl 5-tolylsulfo monoacetone xylose, although all the end-fractions were carefully examined for its presence.

3-Tolylsulfo 5-Benzylcarboxy Monoacetone Xylose—10 gm. of 5-benzylcarboxy monoacetone xylose were dissolved in 50 cc. of pyridine and 6.5 gm. of *p*-tolylsulfonyl chloride were added. The mixture was shaken until the chloride dissolved and was kept in the warm room (at 40°) overnight. The solution was cooled, 2 cc. of water were added, and after 30 minutes at room temperature, more water was added. The product was extracted with chloroform, washed with dilute sulfuric acid, and dried with sodium sulfate. The dry extract was concentrated under reduced pressure to a syrup which was dissolved in warm ether. On cooling, the product crystallized immediately. The crystals were filtered off and a second crop was obtained by adding pentane and recooling. The yield in two crops was 11.3 gm. or 77 per cent of the theoretical, and 2.0 gm. of crude crystalline material were secured by evaporating the mother liquors. The pure fractions were three times recrystallized from 95 per cent alcohol, and the product thus secured melted at 128½–129½°. It had a rotation of $[\alpha]_D^{20} =$

$$\frac{-1.02^\circ \times 100}{2 \times 2.036} = -25.0^\circ \text{ (in chloroform).}$$

The composition agreed with that of a tolylsulfo benzylcarboxy monoacetone pentose.

4.450 mg. substance: 9.450 mg. CO_2 and 2.160 mg. H_2O
0.1036 " " : 0.478 gm. $BaSO_4$
$C_{23}H_{26}O_9S$. Calculated. C 57.72, H 5.48, S 6.70
478.3 Found. " 57.92, " 5.44, " 6.34

The substance is very soluble in acetone and chloroform; quite soluble in benzene and ethyl acetate; almost insoluble in cold, but soluble in hot ether, methyl or ethyl alcohol, hexane, and heptane. It is very slightly soluble in hot or cold pentane or water.

3-Tolylsulfo 5-Benzoyl Monoacetone Xylose from 3-Tolylsulfo 5-Benzylcarboxy Monoacetone Xylose—5 gm. of 3-tolylsulfo 5-benzylcarboxy monoacetone xylose were suspended in 55 cc. of 95 per cent alcohol, approximately 1 gm. of Raney's nickel catalyst was added, and the mixture was hydrogenated at a pressure of 45 pounds per sq. inch, with shaking, for 4 hours. The catalyst was filtered off, and the filtrate concentrated under reduced pressure to a syrup and distilled with toluene to remove traces of water. It was dissolved in 40 cc. of dry pyridine, cooled in ice, and 1.33 cc. of benzoyl chloride (1.1 moles) were added. The mixture was allowed to stand overnight at room temperature, and the product was isolated as usual. The dried chloroform extract was concentrated under reduced pressure to a syrup which was dissolved in ether. After cooling, pentane was added to faint turbidity and the mixture was seeded. In this way there was obtained, in two crops, a total of 4.2 gm. of slightly sticky material. After one recrystallization from methyl alcohol it was substantially pure 3-tolylsulfo 5-benzoyl monoacetone xylose, m.p. 93½–94½°. Mixed melting point with an authentic sample, 94–95°; $[\alpha]_D^{20} = -64.6^\circ$ ($CHCl_3$). The yield of pure product was 3.1 gm. or 66 per cent of the theoretical.

3-Tolylsulfo Xylose—10 gm. of 3-tolylsulfo 5-benzylcarboxy monoacetone xylose were suspended in 100 cc. of dry methyl alcohol and 3 cc. of barium methylate solution (1.3 N) were added. The mixture was shaken 15 minutes until dissolved and was then left at room temperature for 4 hours. It was concentrated under reduced pressure to a syrup and dissolved in 30 cc. of glacial acetic acid. 60 cc. of water and 10 cc. of 5 N sulfuric acid were added and the mixture heated for 2 hours at 80°. Without cooling, a solution of the equivalent quantity (6.3 gm.) of barium acetate was added and the mixture filtered. The filtrate was concentrated under reduced pressure to about half its original volume, filtered with charcoal, and further concentrated. Over calcium chloride in a vacuum desiccator the syrup crystallized spontaneously. The yield of crude crystalline product was 5.6 gm. or 88 per cent of the theoretical. The product was twice recrystallized from isobutyl alcohol and then melted at 123–124° and had a rotation, in absolute alcohol, of $[\alpha]_D^{20} = \frac{+1.61° \times 100}{2 \times 2.004} = +40.2°$. A 2 per cent solution, in water, had specific rotations of +27.1° (1.5 minutes after dissolving), 25.8° (3 minutes), 19.8° (10 minutes), 15.9° (15 minutes), 14.0° (20 minutes), 12.6° (26 minutes), 10.6° (35 minutes), 9.6° (52 minutes), and 9.4° (4 hours).

The composition agreed with that of a tolylsulfo pentose.

4.725 mg. substance: 8.210 mg. CO_2 and 2.250 mg. H_2O
8.765 " " : 6.630 " $BaSO_4$

$C_{12}H_{16}O_7S$.	Calculated.	C 47.34, H 5.30, S 10.54
304.2	Found.	" 47.40, " 5.33, " 10.39

The substance is quite soluble in water and very soluble in methyl and ethyl alcohol, but is fairly insoluble, even at the boiling point, in acetone, ethyl acetate, chloroform, ether, benzene, pentane, hexane, and heptane. It reduces Fehling's solution strongly on warming.

Glycoside Formation of 3-Tolylsulfo Xylose—A 0.0344 M solution of 3-tolylsulfo xylose in methyl alcohol containing 0.5 per cent of dry hydrogen chloride was used for glycoside formation at room temperature and at 76°, the technique previously described[5] being used. The specific rotations are plotted in Fig. 1, in which the curves for 3-methyl xylose are included for comparison. It will be observed that although the types of the curves are identical, the magnitude of the rotation changes is far smaller in the case of the tolylsulfo derivative and the changes are opposite in direction in the two cases.

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[5] Levene, P. A., Raymond, A. L., and Dillon, R. T., *J. Biol. Chem.*, **95**, 699 (1932).

Phospho Xylose from 5-Benzylcarboxy Monoacetone Xylose—To 35 cc. of cold (−40°) dry pyridine were added 4 cc. of phosphorus oxychloride and then, in one portion, a solution of 13 gm. of 5-benzylcarboxy monoacetone xylose in 50 cc. of cold (−30°) pyridine. The mixture was well stirred and then kept for 2 hours in an ice-salt bath. It was recooled to −30° and water was added, drop by drop, until no further heat evolution occurred. Water was added and the mixture was concentrated under reduced pressure until the pyridine was nearly all removed.

60 cc. of glacial acetic acid and 7.2 cc. of 5 N sulfuric acid were now added and the mixture diluted to a volume of 180 cc. It was heated at 80° for 1 hour and then cooled in ice. Freshly prepared silver acetate, in dilute acetic acid, was added until the halide was removed, the mixture was filtered, and the residue washed with acetic acid. The filtrate was treated with hydrogen sulfide and then aerated. The calculated quantity of barium acetate (4.9 gm. of the monohydrate) was added, and the mixture filtered with charcoal. The filtrate was concentrated under reduced pressure and evaporated with addition of water until but little acetic acid remained. It was diluted to about 200 cc. and a solution of neutral lead acetate was added until precipitation was complete. The precipitate was washed several times with dilute acetic acid, and then with water until acid-free.

The lead salt was suspended in water and decomposed with hydrogen sulfide, filtered, refiltered with charcoal, and the filtrate concentrated to about 200 cc. About 5 gm. of Raney's nickel catalyst were added and the mixture hydrogenated overnight under a pressure of about 45 pounds per sq. inch. Toluene was formed, as indicated by the odor. The catalyst was filtered off and washed with water. The filtrate and washings were combined and cooled, barium hydroxide solution was added to about pH 5.6, and the solution was concentrated under reduced pressure to a volume of about 50 cc. It was made alkaline (about pH 8.2) by addition of barium hydroxide, filtered with charcoal, and precipitated with 3 to 4 volumes of acetone. The precipitate was centrifuged off, washed successively with 50 per cent acetone, acetone and ether, and dried over phosphorus pentoxide. The yield was only 1.4 gm.

The composition agreed fairly well with that of the barium salt of a pentose phosphate.

4.780 mg. substance: 28.075 mg. ammonium phosphomolybdate (Pregl)
10.415 " " : 5.885 " $BaSO_4$
$C_5H_9O_8PBa$. Calculated. P 8.50, Ba 37.59
365.5 Found. " 8.53, " 33.25

Rates of Hydrolysis of Xylosephosphoric Esters—An amount of the barium salt of the respective ester to contain 5.00 mg. of phosphorus was dissolved in 50 cc. of 1 N hydrochloric acid. Portions of this solution were sealed in tubes and heated in the steam bath. The barium salts of the esters employed were: (*a*) 3-phospho 5-methyl monoacetone xylose, (*b*) the ester from 5-benzoyl monoacetone xylose, (*c*) the esters from 5-benzylcarboxy monoacetone xylose, (*d*) the esters secured by phosphorylating γ-methyl xyloside, and (*e*) the ester obtained by phosphorylating monoacetone xylose.

The sealed tubes were heated for varying lengths of time, cooled, and opened. The inorganic phosphorus was determined colorimetrically by the method of King,[6] a photoelectric colorimeter being employed for the comparisons. The acid present in the samples was compensated for by adding an exactly equal amount to the calibration standards.

The results are given in Fig. 2, from which it will be seen that with the ester in which the phosphorus is definitely situated in position (3) the rate of hydrolysis is much greater than in the case of any of the others.

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[6] King, E. J., *Biochem. J.*, **26**, 292 (1932).

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THIO SUGARS

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The generally accepted biological significance of substances containing a thiol group is sufficient justification for undertaking a detailed study of the thio sugars. Only one naturally occurring thio sugar in which the thio group is attached to a carbon atom in other than the reducing position is known. This is a thiomethyl pentose which is a component of an adenine nucleoside occurring in yeast. Two papers appeared almost simultaneously on the structure of this sugar; Suzuki, Odake, and Mori[1] assigned to the sugar the structure of a 5-thiomethyl aldopentose, whereas Levene and Sobotka[2] regarded it as a 3- or 4-thiomethyl ketopentose. Neither of these theories appears to have been adequately tested, and further investigation was suggested by Dr. Levene.

In view of the scarcity of the material, the synthetic method of approach seemed most promising. The theory of Suzuki *et al.* is particularly susceptible of investigation by this method, for there are only four possible 5-thiomethyl aldopentoses of either series (*d*- or *l*-) and these would give only two osazones. Thus their theory requires that the osazone of the natural sugar be identical with the osazone of 5-thiomethyl xylose (lyxose) or 5-thiomethyl ribose (arabinose).

The synthesis of 5-thioethyl and 5-thiomethyl xylose was accomplished by heating 5-tolylsulfo monoacetone xylose with the potassium salt of ethyl or methyl mercaptan, followed by hydrolysis of the acetone derivative to the nicely crystalline alkylthio xyloses. By analogy with 5-methyl xylose in method of preparation, these compounds would be expected to have the thio groups attached to carbon atom (5) and this assumption was confirmed by examination of the glycoside formation. The formation of only one glycoside, of the typical γ type, was indicated. The substances may therefore be designated 5-thiomethyl and 5-thioethyl xylose. They rapidly reduce Fehling's solution on warming, and reduce it slowly at room temperature. Since the only ring form in which they can exist is the furanose, it is interesting that these free sugars are so nicely crystalline.

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[1] Suzuki, V., Odake, S., and Mori, T., *J. Agric. Chem. Soc. Japan*, **2**, 1 (1924); *Biochem. Z.*, **104**, 278 (1924).

[2] Levene, P. A., and Sobotka, H. H., *J. Biol. Chem.*, **65**, 551 (1925).

The synthetic 5-thiomethyl xylose differed from the natural sugar in that it was crystalline, had a different specific rotation, and gave a phenylosazone which differed in melting point and optical properties from the osazone of the natural thiomethyl pentose. Thus, 5-thiomethyl xylose, 5-thiomethyl lyxose, and 5-thiomethyl-2-ketoxylose (or -lyxose) are definitely excluded as possible structures for the natural sugar.

On attempting to apply these same methods to the *p*-tolylsulfo 2,3-monoacetone methylribofuranoside described by Levene and Stiller,[3] the reaction apparently followed a different course and after hydrolysis only a small amount of a syrupy reducing substance was obtained. From this material, however, there was prepared a nicely crystalline *p*-bromophenylosazone of correct composition. In its properties this substance also differed from the corresponding osazone of the natural sugar. Had the structure of the syrupy material been definitely established, then position (5) would have been excluded for the allocation of the thiomethyl group in the natural sugar. An attempt was therefore made to prepare 5-thiomethyl ribosazone in a manner that would assure its structure. Since the osazone of 5-thiomethyl arabinose would be identical with that of 5-thiomethyl ribose, it would serve equally well, but unfortunately no derivatives of arabinose which permit substitution in position (5) are easily available. For this reason attention was directed to the possibility of preparing 6-thiomethyl glucose with a view to degrading it to 5-thiomethyl arabinose. The 6-thiomethyl glucose was in fact prepared (from 6-*p*-tolylsulfo monoacetone glucose) and its structure was confirmed by glucoside formation, which was analogous in type to that of glucose and of 6-methyl glucose. The degradation of this derivative to the arabinose compound has not as yet been completed.

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[3] Levene, P. A., and Stiller, E. T., *J. Biol. Chem.*, **106**, 421 (1934).

Because of the further possibility that, in the naturally occurring thio sugar, the thiomethyl group might be attached in a position other than (5), attention was directed to the development of methods for the introduction of thio groups into such positions. The only previous work along this line is that of Freudenberg, who synthesized 3-thiomethyl glucose by isomerizing the xanthate; but this method would be difficult to extend to the substances which we desired to synthesize. In view of the fact that 2-bromo-β-methylglucoside has been shown to give 3-amino-β-methylglucoside on treatment with ammonia, it was hoped that the 3-thiomethyl derivatives might be synthesized analogously. In preliminary experiments, 2-bromo-methylglucoside was treated with potassium methyl mercaptan, giving a thiomethyl hexoside. However, on hydrolysis to the free reducing hexose, it was found that an osazone could not be prepared. Though negative evidence is open to question, it is felt that the experiments were convincing in this regard, and that the thiomethyl group is in all probability attached to carbon atom (2). From the mother liquors of the thiomethyl hexoside preparation, an isomeric, non-crystalline material was isolated by acetylation and distillation. On hydrolysis to the reducing hexose, this material was likewise found to yield no osazone, again indicating substitution in position (2).

In connection with the work on the synthetic thio sugars, their behavior toward oxidizing agents was studied because of its bearing on the possible keto structure of the natural sugar. It was found that the 5-thiomethyl monoacetone xylose, in which the aldehyde group was protected against oxidation, was practically non-reducing in the Lehmann-Maquenne determination, indicating no oxidation of the thio group by this reagent. In the Willstätter determination, on the other hand, exactly 1 atom of oxygen was used, indicating quantitative oxidation of the CH_3S— to CH_3SO—. The free thiomethyl xylose required 1.92 atoms of oxygen in the Willstätter oxidation so that both aldehyde and thio groups are almost quantitatively oxidized. In the Lehmann-Maquenne determination the free thio sugar used 1.39 atoms of oxygen, 1 mg. of the thio pentose being equivalent to 0.42 mg. of glucose.

EXPERIMENTAL

5-Thioethyl Monoacetone Xylose—12 gm. of 5-*p*-tolylsulfo monoacetone xylose and 6.0 gm. (2 moles) of dry sodium ethyl mercaptan were added to 70 cc. of dry acetone and sealed in a Pyrex flask body. The mixture was heated in the boiling water bath for 2 hours, cooled, and opened. Without filtering, the mixture was concentrated under reduced pressure, water being added from time to time to keep the sodium toluenesulfonate in solution. The aqueous solution was extracted with 600 cc. of chloroform in three portions, the extract washed with dilute, ice-cold sulfuric acid, and then three times with ice water. The chloroform extracts were dried with sodium sulfate and concentrated under reduced pressure to a thick syrup. This was dissolved in hot hexane and cooled in a solid carbon dioxide-alcohol bath. The product crystallized on stirring. It was washed with cold hexane and air-dried. By reworking the mother liquors a second crop was secured, the total yield of air-dry material being 6.1 gm. or 75 per cent of the theoretical. The product was three times recrystallized from hexane and it then melted at 66½–67½° and had a rotation, in absolute alcohol, of

$$[\alpha]_D^{22} = \frac{-2.35° \times 100}{2 \times 2.04} = -57.5°$$

The composition agreed with that of a thioethyl monoacetone pentose.

4.585 mg. substance: 8.600 mg. CO_2 and 3.100 mg. H_2O
8.164 " " : 8.030 " $BaSO_4$
7.585 " " : 7.570 " AgI
$C_{10}H_{18}O_4S$. Calculated. C 51.24, H 7.75, S 13.69, OC_2H_5 19.21
234.2 Found. " 51.15, " 7.57, " 13.50, " 19.14

The substance is very soluble in methyl or ethyl alcohol, ether, acetone, ethyl acetate, and chloroform; fairly soluble in hot water, boiling heptane, or hexane; little soluble in boiling pentane; and almost insoluble in water, heptane, hexane, and pentane in the cold.

5-Thioethyl Xylose—2 gm. of 5-thioethyl monoacetone xylose were added to 40 cc. of 1 N H_2SO_4 and the mixture was heated at 50° with frequent shaking until dissolved (about 1 hour). The solution was then heated to 80°, maintained at this temperature for 2 hours, cooled, and shaken with an excess of barium carbonate. The mixture was filtered and the filtrate evaporated to dryness in a vacuum desiccator over phosphorus pentoxide. The product crystallized spontaneously. It was extracted several times with boiling ether, leaving a small syrupy residue, which was discarded. The ether solutions were concentrated to a small volume and cooled. On seeding and stirring, the product crystallized. A second crop was obtained from the mother liquors, the yield of crystalline material being 1.4 gm. or 84 per cent of the theoretical. The material was recrystallized from ether in the same manner and then melted at 81–82° and had a rotation, in absolute alcohol, of

$$[\alpha]_D^{22} = \frac{-1.69° \times 100}{2 \times 2.00} = -42.2°$$

3 minutes after dissolving and was unchanged after 2 hours. The rotation, in water, was

$$[\alpha]_D^{22} = \frac{+1.20° \times 100}{2 \times 2.012} = +29.8°$$

1 minute after dissolving, +20.6° (2 minutes), +17.1° (3 minutes), +16.4° (5 minutes), +15.4° (10 minutes, 20 minutes, and 1½ hours).

The composition agreed with that of a thioethyl pentose.

4.030 mg. substance: 6.450 mg. CO_2 and 2.710 mg. H_2O
10.840 " " : 12.820 " $BaSO_4$
8.471 " " : 9.495 " AgI
$C_7H_{14}O_4S$. Calculated. C 43.26, H 7.27, S 16.51, OC_2H_5 23.17
194.2 Found. " 43.65, " 7.53, " 16.24, " 21.48

The substance is very soluble in water, methyl or ethyl alcohol, chloroform, and acetone. It is fairly soluble in hot ether or ethyl acetate, but not very soluble in the cold. It is very little soluble even at the boiling point, in heptane, hexane, or pentane.

5-Thiomethyl Monoacetone Xylose—15 gm. of 5-*p*-tolylsulfo monoacetone xylose, 7.6 gm. (2 moles) of dry potassium methyl mercaptan, and 75 cc. of dry acetone were heated, in a sealed flask, in the boiling water bath for 4 hours. The subsequent procedure was the same as for the ethyl derivative. The washed, dried chloroform extracts were concentrated under reduced pressure to a syrup which was dissolved in hexane. The product crystallized immediately and was filtered off and washed with hexane. By reworking the mother liquors, two additional crops of material were secured, the total weight of air-dry substance being 7.2 gm. (75 per cent of the theoretical). The product was recrystallized from hexane containing 8 per cent of ethyl acetate, and then twice recrystallized from hexane containing 5 per cent of ethyl acetate. The material then melted at 91½–92° and had a rotation, in absolute alcohol, of

$$[\alpha]_{D}^{22} = \frac{-2.20° \times 100}{2 \times 2.04} = -53.9°$$

The composition agreed with that of a thiomethyl monoacetone pentose.

5.244 mg. substance: 9.500 mg. CO_2 and 3.480 mg. H_2O
10.912 " " : 11.522 " $BaSO_4$
6.990 " " : 7.580 " AgI

$C_9H_{16}O_4S$.	Calculated.	C 49.05,	H 7.33,	S 14.56,	OCH_3 14.07
220.2	Found.	" 49.41,	" 7.43,	" 14.50,	" 14.32

The solubilities of the substance are similar to those of the corresponding ethyl derivative.

5-Thiomethyl Xylose—2 gm. of 5-thiomethyl monoacetone xylose were added to 40 cc. of 1 N H_2SO_4, heated to 50°, and shaken frequently until dissolved (about 30 minutes). The subsequent treatment was the same as for the thioethyl derivative. The yield of crude crystalline product was 1.4 gm. (85 per cent of the theoretical). The product, twice crystallized from ether, had a melting point of 74–75°. The rotation, in absolute alcohol, was

$$[\alpha]_{D}^{20} = \frac{-0.46° \times 100}{2 \times 1.0} = -23.0°$$

3 minutes after dissolving and −39.0° at equilibrium. In water it was

$$[\alpha]_{D}^{20} = \frac{+1.45° \times 100}{2 \times 1.992} = +36.4°$$

1 minute after dissolving, 27.8° at 2 minutes, 22.8° at 3 minutes, 22.6° at 4½ minutes, 22.1° at 6 minutes, 20.6° at 9 minutes, 24 minutes, and 3 hours.

The composition agreed with that of a thiomethyl pentose.

5.018 mg. substance: 7.370 mg. CO_2 and 3.100 mg. H_2O
7.220 " " : 9.290 " $BaSO_4$
5.390 " " : 6.740 " AgI
$C_6H_{12}O_4S$. Calculated. C 39.96, H 6.72, S 17.79, OCH_3 17.20
180.4 Found. " 40.06, " 6.92, " 17.67, " 16.51

The solubilities of the substance are similar to those of the corresponding thioethyl derivative.

5-Thiomethyl Xylosazone—0.5 gm. of crude crystalline 5-thiomethyl xylose was dissolved in water and 1.0 cc. (3.3 moles) of phenylhydrazine dissolved in 1 cc. of glacial acetic acid was added. The mixture was heated on the steam bath for 2 hours and cooled. The osazone was filtered off, washed with methyl alcohol, and then recrystallized from methyl alcohol. The final material was a felt of coarse lemon-yellow needles. The composition corresponded to that of a thiomethyl pentosazone.

4.712 mg. substance: 0.633 cc. N (23° and 766 mm.)
7.400 " " : 4.730 mg. $BaSO_4$
$C_{18}H_{22}O_2SN_4$. Calculated. N 15.65, S 8.94
358.3 Found. " 15.62, " 8.78

The product sintered slightly at 151° and melted without decomposition at 162–163°. The rotation in pyridine-absolute alcohol (2:3 by volume) was

$$[\alpha]_D^{22} = \frac{-0.17° \times 100}{0.5 \times 1.036} = -32.8°$$

2 minutes after dissolving, and −21.2° at equilibrium. The osazone from the natural product was reported by Levene and Sobotka[2] as having an initial rotation in the above solvent of +6° and +7° at equilibrium. A newly prepared sample of the osazone of the natural product was found to melt at 160–161° and to have a mixed melting point with the osazone of the synthetic material of 143–145°. Thus there can be no doubt as to the non-identity of the two osazones.

Glycoside Formation on 5-Thiomethyl and 5-Thioethyl Xylose—The method and technique were those previously employed, the glycoside formation being conducted at room temperature and at 76°. The results are plotted in Fig. 1, 5-methyl xylose being included for comparison. It will be seen that in all cases only one glycoside appears to be formed and that this is of the typical γ type.

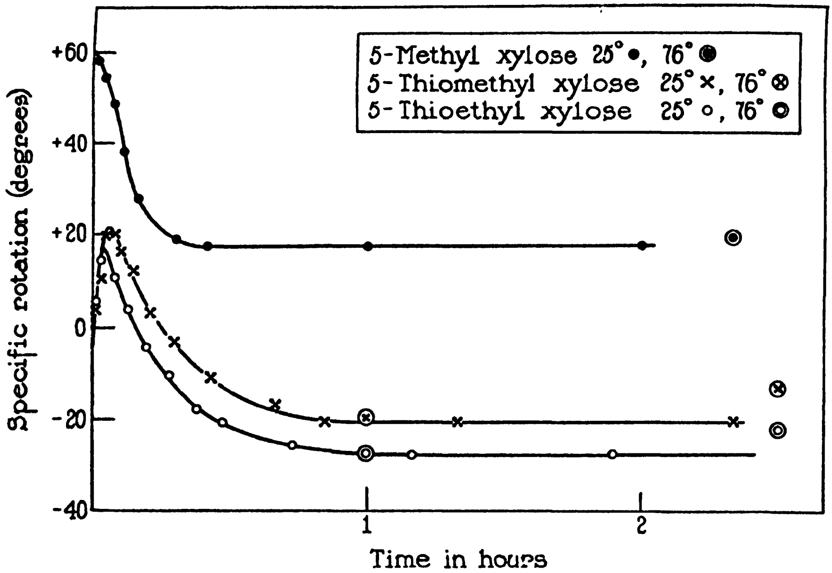

FIG. 1. Glycoside formation on 5-thiomethyl, 5-thioethyl, and 5-methyl xylose.

TABLE I

Oxidation Equivalents of 5-Thiomethyl Xylose and 5-Thiomethyl Monoacetone Xylose

Method	Substance	Amount of substance	0.1 N thiosulfate	Atoms O per mole	Glucose equivalent to 1 mg. thio sugar
		mg.	*cc.*		*mg.*
Lehmann-Maquenne	Thiomethyl xylose	7.97	1.22	1.39	0.42
"	" monoacetone xylose	50.7	0.04	0.01	
Willstätter	" xylose	31.8	6.76	1.92	
"	" monoacetone xylose	50.7	4.63	1.00	

Oxidation Determinations—The thiomethyl xylose and its monoacetone derivative were used for oxidation determinations by both the Lehmann-Maquenne and Willstätter methods. The results are given in Table I.

6-Thiomethyl Monoacetone Glucose—45 gm. of 6-tolylsulfo monoacetone glucose and 22 gm. of dry potassium methyl mercaptan were added to 200 cc. of dry, peroxide-free dioxane, and sealed in a Pyrex flask body. The mixture was shaken overnight at room temperature and was then heated in the steam bath, with occasional shaking, for 1¼ hours. The mixture was cooled, the bulb opened, and considerable ether was added. The precipitate was filtered off and extracted several times with boiling ether. The ethereal filtrate and extracts were combined, concentrated under reduced pressure to a small volume, and part of the dioxane was distilled off. 800 cc. of ether were added to the residue, the solution filtered with charcoal, and concentrated under reduced pressure to a syrup. This was distilled (at about 0.1 mm.), the boiling point being 140–155°. Four fractions (total weight 23.3 gm.) were obtained and of these the third crystallized spontaneously. When this was used for nucleation, all the fractions crystallized to a hard mass; but recrystallization from a solvent has not as yet been effected. The second fraction had a rotation, in absolute alcohol, of

$$[\alpha]_{\mathrm{D}}^{22} = \frac{-0.16^\circ \times 100}{2 \times 2.282} = -3.5^\circ$$

and the last fraction, in the same solvent, a rotation of −7.6°.

The composition of the product agreed with that of a thiomethyl monoacetone hexose.

4.498 mg. substance: 7.920 mg. CO_2 and 2.810 mg. H_2O
7.180 " " : 6.648 " $BaSO_4$
5.840 " " : 5.485 " AgI
$C_{10}H_{18}O_5S$. Calculated. C 47.96, H 7.25, S 12.81, OCH_3 12.39
250.2 Found. " 48.02, " 7.00, " 12.72, " 12.40

6-Thiomethyl Glucose—5 gm. of 6-thiomethyl monoacetone glucose were added to 30 cc. of 1 N sulfuric acid, the mixture warmed and shaken until dissolved, and then heated at 80° for 2 hours.

The solution was cooled, the sulfuric acid neutralized with barium carbonate, and the filtrate concentrated under reduced pressure to a syrup. Despite repeated attempts, crystallization of this syrup has not as yet been effected. It strongly reduced hot Fehling's solution.

1 gm. of this syrup was heated on the steam bath with 3 moles of *p*-bromophenylhydrazine and acetic acid, and the osazone which formed was filtered off. A second crop was secured by heating for a further time and then cooling in the refrigerator. The combined material was extracted twice with hot methyl alcohol in which the osazone is practically insoluble. It was then a felt of beautiful, yellow needles. It darkened, on rapid heating, at 177° and melted at 184–185°. The rotation, in pyridine-absolute alcohol (3:2 by volume) was

$$[\alpha]_D^{20} = \frac{-0.24° \times 100}{0.5 \times 1.00} = -48°$$

2 minutes after dissolving, and $-16°$ at equilibrium after 5 hours.

The composition agreed with that of a bromophenylosazone of a thiomethyl hexose.

4.600 mg. substance: 7.060 mg. CO_2 and 1.650 mg. H_2O
6.299 " " : 0.564 cc. N (at 27° and 759.5 mm.)
$C_{19}H_{22}O_3N_4Br_2$. Calculated. C 41.75, H 4.06, N 10.26
546.1 Found. " 41.86, " 4.02, " 10.19

Glucoside Formation on 6-Thiomethyl Glucose—The method and technique were identical with those for the xylose derivative. The results are plotted in Fig. 2, 6-methyl glucose being included for comparison.

Thiomethyl Methylhexoside from 2-Bromo-β-Methylglucoside I—Bromine was added to triacetylglucal, and the bromo sugar converted to the glucoside, substantially as described by Fischer *et al.*[4] The first form crystallized in good yield, was recrystallized, and deacetylated.

30 gm. of dry, recrystallized 2-bromo-methylglucoside I and 15 gm. of dry potassium methyl mercaptan were added to 350 cc. of dry acetone and sealed in a Pyrex flask body. This was heated in

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[4] Fischer, E., Bergmann, M., and Schotte, H., *Ber. chem. Ges.*, **53,** 509 (1920).

the steam bath for 5 hours, cooled, and opened. The insoluble material was filtered off and washed several times with hot acetone, the combined acetone solutions were concentrated as far as possible, and considerable chloroform was added. The mixture was boiled for several minutes and filtered hot. The residue was washed with hot chloroform, and the combined solutions were concentrated under reduced pressure to a syrup. This material crystallized spontaneously on standing. It was triturated with acetone to remove the syrupy fraction and the crystalline material which remained weighed, on drying, 8.6 gm. It was recrystallized from 45 volumes of hot acetone. After two recrystallizations it had a melting point of 128½–129½° and a rotation, in water, of

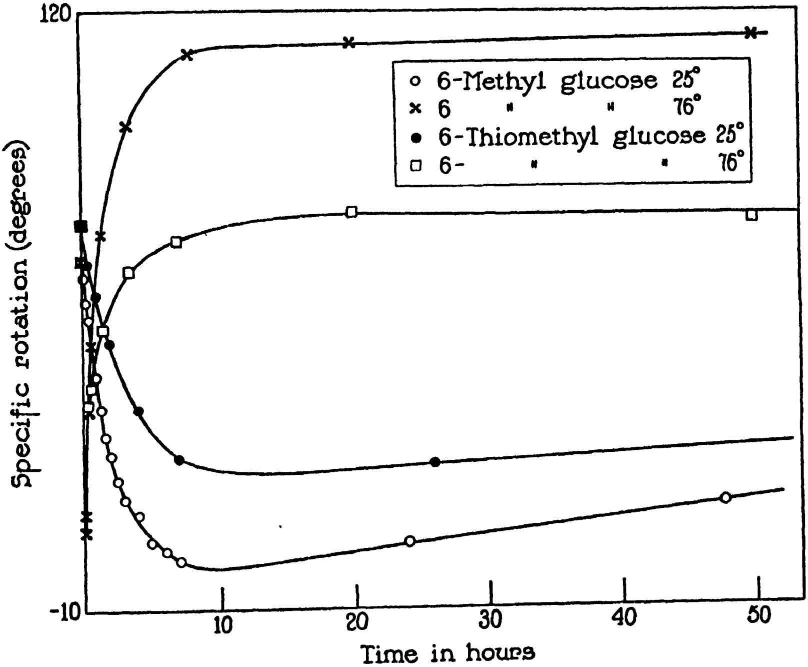

FIG. 2. Glucoside formation on 6-thiomethyl and 6-methyl glucose

$$[\alpha]_D^{20} = \frac{-2.09° \times 100}{2 \times 1.988} = -52.6°$$

The composition agreed with that of a thiomethyl methylhexoside.

4.600 mg. substance: 7.220 mg. CO_2 and 2.930 mg. H_2O
9.150 " " : 59.505 " $BaSO_4$
5.470 " " : 10.970 " AgI
$C_8H_{16}O_5S$. Calculated. C 42.82, H 7.19, S 14.30, OCH_3 27.65
224.2 Found. " 42.81, " 7.13, " 14.28, " 26.48

A portion of this material was hydrolyzed with 1.7 N hydrochloric acid until the rotation became constant. The solution was not very strongly reducing and no osazone was formed on heating with either phenylhydrazine or *p*-bromophenylhydrazine. The non-crystalline mother liquors resulting from the isolation of the thiomethyl methylhexoside were combined, acetylated, and distilled under greatly reduced pressure. The syrup was deacetylated and hydrolyzed with hydrochloric acid until the rotation became constant. The solution was slightly reducing but no osazone was obtained on heating with either phenylhydrazine or *p*-bromophenylhydrazine.

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ISOBUTYRYLFORMIC ACID PHENYLHYDRAZONE

BY LYMAN C. CRAIG

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, June 28, 1934)

In an earlier paper[1] we have reported the isolation of isobutyrylformic acid from the reaction mixture which results on treatment of ergotinine with aqueous alkali. The phenylhydrazone of the ketonic acid was prepared for final identification. Although the analytical figures obtained with this derivative were satisfactory, its melting point (152°) proved to be higher than that recorded in the literature (128°[2] and 143°[3]). In order to clear up this discrepancy, we have since prepared the phenylhydrazone of synthetic isobutyrylformic acid. The latter melts at 152°, thus confirming our observation on the substance obtained from ergotinine. A mixed melting point showed no depression.

The method of synthesis was essentially that reported by Tschelinzeff[4] with a slight variation in the method of hydrolysis of the intermediate isobutyryl cyanide. It was found necessary to heat the cyanide on the steam-bath for two hours in 1:1 hydrochloric acid in order to effect hydrolysis. The hydrolysis mixture was extracted with ether, the ether extract dried with sodium sulfate and evaporated to a sirup. If this sirup is used to prepare the hydrazone it does not crystallize. However, if the sirup is shaken with a little water, in which it is not completely soluble, and the aqueous layer used to prepare the hydrazone, the product crystallizes nicely. Repeated recrystallizations from dilute alcohol failed to raise the melting point above 152°.

Anal. Calcd. for $C_{11}H_{14}O_2N_2$: C, 64.08; H, 6.84; N, 13.59. Found: C, 64.15; H, 6.74; N, 13.66.

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(1) W. A. Jacobs and L. C. Craig, *J. Biol. Chem.*, **104**, 547 (1934).

(2) D. Tschtschenke, *Bull. soc. chim.*, [4] **37**, 623 (1925).

(3) E. Abderhalden and E. Rossner, *Z. physiol. Chem.*, **163**, 261 (1926).

(4) W. Tschelinzeff and W. Schmidt, *Ber.*, **62**, 2210 (1929).

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, October, 1934,
Vol. 107, No. 1, pp. 143-154]

STROPHANTHIN

XXXI. FURTHER STUDIES ON THE DEHYDROGENATION OF STROPHANTHIDIN

BY ROBERT C. ELDERFIELD AND WALTER A. JACOBS

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, July 23, 1934)

In a recent preliminary note[1] of our later studies of the dehydrogenation of strophanthidin with selenium we briefly reported the formation of Diels' hydrocarbon, methylcyclopentanophenanthrene, thus confirming a similar observation made by Tschesche and Knick[2] with a derivative of another cardiac aglucone, dianhydrouzarigenin. In the present paper we wish to describe these experiments more fully with resulting conclusions. We shall refer the reader to the preliminary note for the historical points.

In the first work of Jacobs and Fleck,[3] which yielded a substance that appeared to be a dimethylphenanthrene, $C_{16}H_{14}$, a procedure had been used which in preliminary trials with cholesteryl chloride had given the Diels hydrocarbon, $C_{18}H_{16}$, without difficulty. In this procedure the mixture of strophanthidin and selenium was heated in a Pyrex flask furnished with an outer jacket containing mercury refluxing at ordinary pressure.

The temperature at which the dehydrogenation occurred was thus 350–360°, or possibly somewhat higher if superheating had occurred. In the present work a much stricter control of temperature conditions has been observed by the use of a potassium sodium nitrate bath equipped with a thermometer. Under the conditions given in the experimental part and at a final temperature of 320–340°, the Diels hydrocarbon has been obtained without difficulty from the reaction mixture. Its identity has been confirmed by preparation of the picrate, trinitrobenzene, and trinitrotoluene addition compounds. Further, on oxidation with chromic acid no quinone was obtained. The possibility that the $C_{16}H_{14}$ hydrocarbon of Jacobs and Fleck might be contained in the mother liquors or fractions other than those which gave the Diels hydrocarbon was then considered. However, oxidation experiments with various fractions of the hydrocarbons failed to yield any of the previously described quinone obtained from the $C_{16}H_{14}$ hydrocarbon.

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[1] Elderfield, R. C., and Jacobs, W. A., *Science*, **79**, 279 (1934).

[2] Tschesche, R., and Knick, H., *Z. physiol. Chem.*, **222**, 58 (1934).

[3] Jacobs, W. A., and Fleck, E. E., *J. Biol. Chem.*, **97**, 57 (1932).

The problem, therefore, remained of reconciling the earlier described production of the $C_{16}H_{14}$ hydrocarbon with our failure to obtain any appreciable quantities of this substance in the later work which gave the Diels hydrocarbon. The thought naturally occurred that the discrepancies might be explained by the differences in the temperatures at which the dehydrogenation was carried out as well as in the rates of heating and rates of addition of the selenium in the two cases. That such factors can affect the course of dehydrogenation, with consequent variation in the products obtained, has been shown by the work of Ruzicka and coworkers on the dehydrogenation of cholic acid and the sterols.[4] To check such possibilities, other experiments were performed.

Strophanthidin was heated rapidly at 340–360° without addition of selenium until decomposition had substantially ceased and a quiet melt had been produced. Excess selenium was then added and the mixture was heated for 32 hours at this temperature. After fractional distillation and prolonged fractional crystallization, only a very small amount of hydrocarbon melting at 123–124° was obtained. This substance did not appear homogeneous under the fluorescent microscope and on treatment with picric acid gave an unhomogeneous picrate which melted neither sharply nor constantly. Upon oxidation of the hydrocarbon a very small quantity of quinone was obtained. In this case the yield of crystalline hydrocarbons was much smaller than in the experiment which gave the Diels hydrocarbon readily.

Finally, strophanthidin was mixed with excess selenium and rapidly heated to 340–360°. This temperature was maintained for 32 hours. In this case, after distillation the total yield of hydrocarbons which crystallized was strikingly large and amounted to about one-half of the weight of strophanthidin taken. Repeated fractional crystallization gave at least four different hydrocarbons.

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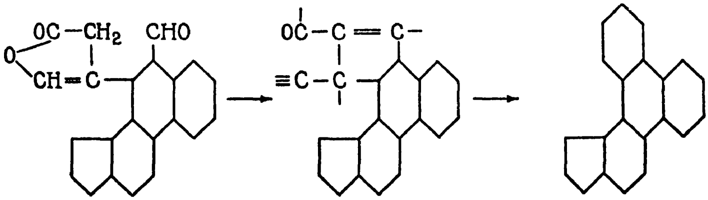
[4] Ruzicka, L., Thomann, G., Brandenberger, E., Furter, M., and Goldberg, M. W., *Helv. chim. acta*, **17**, 200 (1934).

The most insoluble fraction melted constantly at 295–297°. The analytical figures were in good agreement with those required by a $C_{21}H_{16}$ hydrocarbon. It is barely possible that this hydrocarbon is a cyclopentanotriphenylene. This might be formed by a pyrolytic synthesis involving preliminary condensation of the aldehyde group of the strophanthidin molecule on the α-carbon atom of the lactone side chain, to form an intermediate cyclopentene ring and subsequent dehydrogenation. The new methylcyclopentene ring could subsequently rearrange to a benzene ring. However, this can be presented at the moment as a mere conjecture. Such a synthesis is represented in the accompanying formulas.



From the more soluble fractions, hydrocarbon material which melted at 120–121° was obtained. From this substance on oxidation a small quantity of quinone was obtained. However, on treatment with trinitrobenzene approximately one-half of the quantity of hydrocarbon used could be isolated as the trinitrobenzene derivative of Diels' hydrocarbon. Attention was thus concentrated on the most soluble hydrocarbons still remaining in the mother liquor of the above hydrocarbon fraction. From these, by fractional crystallization of the picrates and regeneration from them of the hydrocarbons, it has been possible to isolate two substances. The first hydrocarbon gave analytical figures corresponding to $C_{16}H_{14}$ and melted at 123–124°. It formed a rusty orange picrate melting at 140–141°. On oxidation, a quinone was obtained which melted at 208–209° and which with *o*-phenylenediamine gave a quinoxaline melting at 150°. The second hydrocarbon also gave analytical figures corresponding to $C_{16}H_{14}$.

It melted at 127°, forming a yellow-orange picrate melting at 126–127°, and a trinitrobenzene compound melting at 151–153°. On oxidation of the very small amount available, a quinone was obtained which melted at 203–205°, which in turn gave a quinoxaline, the melting point of which reached 168–171° after several recrystallizations. However, at this point, exhaustion of material prevented further recrystallization.

Unfortunately, owing to the limited amount of material available and the large losses involved in the separation of such complex mixtures of substances possessing similar physical properties, the quantities of hydrocarbons finally obtained were so small that a complete characterization of their quinones and quinoxalines could not be achieved. The results reported are, therefore, not final. Sufficient information has, however, been gathered to show that under these conditions of dehydrogenation there are formed along with the Diels hydrocarbon, at least two other substances possessing very close physical resemblances to it. These may perhaps be provisionally regarded as dimethyl- or ethylphenanthrenes. In the earlier work of Jacobs and Fleck the substance there reported was apparently a mixture of these three hydrocarbons, and, on preparation of derivatives, naturally the most insoluble one was obtained in each case.

An explanation for the varying products obtained in the dehydrogenation of strophanthidin would seem to be found in the order and manner in which the various hydroxyl groups are removed at the beginning of the reaction, since the work of Ruzicka and Thomann[5] has shown that, when once formed, Diels' hydrocarbon appears to resist the further dehydrogenating action of selenium.

The production of Diels' hydrocarbon by the dehydrogenation of strophanthidin and dianhydrouzarigenin leads to the conclusion that, in so far as the formation of this substance may be considered characteristic of the reduced cyclopentanophenanthrene ring system of the sterols, bile acids, etc., the cardiac aglucones appear to be built on this same system. Whether Diels' hydrocarbon is always a primary product, or whether it can also be the result of pyrolytic rearrangements, is a question which must be left for future work. Although the so called $C_{16}H_{14}$ hydrocarbons may be regarded as homologues of phenanthrene, their formation would first have to be proved to be of primary character before the presence of a phenanthrene nucleus in the strophanthidin molecule can be regarded as conclusively proved.

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[5] Ruzicka, L., and Thomann, G., *Helv. chim. acta*, **16**, 226 (1933).

Uzarigenin contains a methyl group in the position occupied by the aldehyde group of strophanthidin, and these groups have been shown to be attached to a $>$CH group of a six-membered ring. The synthetic work of Cook and Haslewood,[6] Haworth, Mavin, and Sheldrick,[7] Harper, Kon, and Ruzicka[8] would indicate that while quaternary methyl groups are eliminated during the course of dehydrogenation, those directly attached to a single ring appear to be much more stable. The ultimate fate, therefore, of this aldehyde carbon atom of strophanthidin becomes of interest. For since this methyl group is attached to a six-membered ring, then the expected dehydrogenation product should be a dimethylcyclopentanophenanthrene and not Diels' hydrocarbon. However, the production of the former substance has not been detected. The apparent elimination, therefore, of the aldehydic carbon atom in strophanthidin (or methyl group of uzarigenin) during the course of its dehydrogenation does not, however, cause serious difficulty, since recent work of Haworth *et al.*[7] has demonstrated possible migration of substituents during such reactions. The necessity is quite evident for caution in the interpretation of the results of dehydrogenation experiments performed at high temperature.

Further, on the mere chance that in spite of accumulated evidence to the contrary, the aldehyde group of strophanthidin might be situated on a five-membered ring, and therefore the source of the methyl group of Diels' hydrocarbon, a study was made on one of its derivatives from which this particular carbon atom could first be removed. This substance is isostrophanthic acid which loses this carbon atom (carbon atom (1)) as CO_2 upon thermal decomposition.[9] Isostrophanthic acid after such preliminary thermal decomposition was heated at 320–340° with selenium for 33 hours. From the reaction mixture the only apparently pure substance isolated was a hydrocarbon ($C_{16}H_{14}$?) melting at 140–142°, which was further characterized by its trinitrobenzene addition product. From the mixtures with melting points near that of the $C_{18}H_{16}$ or "$C_{16}H_{14}$" hydrocarbons no pure substance could be obtained after more than 250 recrystallizations. The results of this work were, therefore, inconclusive in so far as evidence of the formation of cyclopentanophenanthrene is concerned.

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[6] Cook, J. W., and Haslewood, G. A. D., *J. Chem. Soc.*, 428 (1934), and earlier papers.

[7] Haworth, R. D., Mavin, C. R., and Sheldrick, G., *J. Chem. Soc.*, 454 (1934), and earlier papers.

[8] Harper, S. H., Kon, G. A. R., and Ruzicka, F. C. J., *J. Chem. Soc.*, 124 (1934), and earlier papers.

EXPERIMENTAL

Dehydrogenation of Strophanthidin Leading to Diels' Hydrocarbon —150 gm. of strophanthidin and 30 gm. of selenium were placed in a long necked flask equipped with a ground-in tube for sweeping the interior with nitrogen to facilitate the removal of water. The flask was heated in a potassium and sodium nitrate bath. During the first 6 hours the temperature was gradually raised to 320° with further addition of selenium in 30 gm. portions at 2 hour intervals. The temperature was then maintained at 320–340° for an additional 26 hours, with gradual addition of more selenium in portions until a total of 220 gm. had been added. The contents of the flask were finely powdered and extracted with ether in a Soxhlet apparatus. After removal of the ether, the extracted tar was distilled at 1 mm. and cuts were taken as follows:

Fraction I, below 170°, 5 gm.; Fraction II, 170–210°, 6 gm.; Fraction III, 210–240°, 47.3 gm.; Fraction IV, 240–275°, 13.7 gm. None of these fractions crystallized on standing in the refrigerator. Fraction III was redistilled at 0.2 mm. and the following cuts were taken.

Fraction III-a, below 195°, 17.8 gm.; Fraction III-b, 195–220°, 15 gm. Fraction III-a, deposited crystals on standing in the refrigerator, but Fraction III-b remained a glossy resin. Fraction III-a was converted into the picrate in ethereal picric acid and evaporated to dryness. After extraction of the residue with petroleum ether, the undissolved picrates were decomposed with Na_2CO_3 and the hydrocarbons thus obtained were fractionally recrystallized from alcohol according to the triangle scheme. After about forty-five crystallizations, a fraction of 500 mg. was obtained, which melted at 125–126° (corrected). Further recrystallization failed to change the melting point. Under the fluorescent microscope the substance exhibited a strong blue fluorescence and appeared to be contaminated with but a trace of material possessing a lavender fluorescence ($C_{16}H_{14}$?). It yielded no quinone on oxidation.

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[9] Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **61**, 399 (1924).

$C_{18}H_{16}$. Calculated. C 93.10, H 6.89
Found. " 93.12, " 7.05
" 92.98, " 7.11

The picrate was prepared in alcoholic picric acid. It formed yellow-orange needles which melted at 118–119° after recrystallization from alcoholic picric acid.

$C_{24}H_{19}O_7N_3$. Calculated. C 62.44, H 4.16
Found. " 62.07, " 4.01

The trinitrobenzene compound was prepared in alcoholic solution and formed yellow needles which melted at 148–150° after recrystallization from alcohol.

$C_{24}H_{19}O_6N_3$. Calculated. C 64.69, H 4.31
Found. " 64.40, " 4.07

The trinitrotoluene compound formed yellow needles from alcohol and melted at 93–94°.

$C_{25}H_{21}O_6N_3$. Calculated. C 65.34, H 4.61
Found. " 65.50, " 4.57

The higher boiling fractions furnished but very small amounts of crystalline hydrocarbons.

Oxidation experiments with chromic acid on lower melting hydrocarbon fractions obtained from the mother liquors of Diels' hydrocarbon yielded but traces of possible quinones and in amounts too small for investigation. A similar experience was encountered with the higher boiling crystalline hydrocarbon fractions.

Dehydrogenation of Strophanthidin after Preliminary Decomposition—75 gm. of strophanthidin were heated as rapidly as possible to 340–360° in a flask of the type described above. After decomposition and frothing had ceased, a quiet melt remained. 90 gm. of selenium were then added and the mixture was held at 320–340° for 32 hours. After being worked up as in the preceding experiment, the mixture was distilled at 0.2 mm. and the following fractions were taken: Fraction I, up to 175°, 1.7 gm.; Fraction II, 175–200°, 7.0 gm.; Fraction III, 200–225°, 7.5 gm.; Fraction IV, 225–260°, 6.02 gm.; residue, 25 gm. Fraction II crystallized for the most part on standing in the refrigerator. The others remained viscous oils or varnishes. The crystalline material was worked through the picrate and the regenerated hydrocarbon was fractionally crystallized from alcohol according to the triangle scheme. After about 50 recrystallizations a small fraction melted constantly at 123–124°. On oxidation a very small amount of quinone was formed. The picrate melted neither sharply nor constantly at 128–130° and the trinitrobenzene compound melted at 150–152°. The substance was apparently a mixture of hydrocarbons, but the quantity available was too small for proper investigation. From the higher boiling hydrocarbons no crystalline material could be isolated.

Dehydrogenation of Strophanthidin Leading to Diels' Hydrocarbon and the $C_{16}H_{14}$ (?) Hydrocarbons—80 gm. of strophanthidin were intimately mixed with 120 gm. of selenium and the mixture was heated to 340° as rapidly as possible (20 minutes). The temperature of the bath was maintained at 340–360° for 32 hours. After extraction of the residue with ether, the mixture of hydrocarbons was distilled at 0.2 mm. and cuts were taken as follows: Fraction I, up to 150°, 0.4 gm. of oil; Fraction II, 150–215°, 21.6 gm. (crystallized on standing); Fraction III, 215–280°, 7.3 gm. (crystallized on standing); residue, 12 gm. Fraction II was redistilled at 0.2 mm. After discarding a slight oily fore-run, cuts were taken as follows: Fraction II-a, 170–185°, 15 gm.; Fraction II-b, 185–215°, 4.5 gm. Fraction II-a was converted into a picrate, and after extraction of the latter with petroleum ether, the hydrocarbon was regenerated and fractionally recrystallized from alcohol by the triangle scheme. Fraction II-b was crystallized from alcohol directly and the fractions after three recrystallizations united with those of similar melting point from Fraction II-a.

Finally, after removal of considerable higher melting mixtures a fraction of 700 mg. melting at 120–121° was obtained after about 60 recrystallizations. Inasmuch as this still was obviously a mixture, further attempts at resolution were discontinued and oxidation experiments begun on this material. In several oxidation experiments under varying conditions a very small quantity of quinone was obtained which was insufficient for purification. However, a portion of this hydrocarbon mixture furnished a trinitrobenzene compound which melted after repeated recrystallization at 147–148° and represented about one-half of the material taken. The analytical figures corresponded to the trinitrobenzene compound of Diels' hydrocarbon. Found, C 64.65, H 4.06. On conversion of a small sample of this hydrocarbon mixture into the picrate a product was obtained which, after several recrystallizations from alcoholic picric acid, formed rusty orange plates which melted at 125–126° and therefore at a higher temperature than the picrate of Diels' hydrocarbon (118°). Further recrystallization would doubtless have raised this melting point still more, but the small amount of material at hand prevented further recrystallization. On analysis the sample gave figures corresponding to the picrate of a $C_{16}H_{14}$ hydrocarbon.

$C_{22}H_{17}O_7N_3$. Calculated. C 60.66, H 3.95
Found. " 60.60, " 3.94

Attention was then concentrated on the relatively large amounts of lower melting hydrocarbons contained in the mother liquors of the above 700 mg. fraction. The fraction immediately following the latter was converted into a picrate which was then recrystallized from alcoholic picric acid to a constant melting point of 126–127°. The hydrocarbon recovered from the picrate melted constantly at 127° after three recrystallizations from alcohol.

$C_{16}H_{14}$. Calculated. C 93.15, H 6.85
Found. " 92.76, " 6.87

The picrate prepared from the pure substance formed lemon-yellow needles and melted again at 126–127° after recrystallization from alcoholic picric acid. Found, C 61.34, H 4.09.

The trinitrobenzene derivative proved to be more stable than the picrate and formed lemon-yellow needles which melted at 151–153° after recrystallization from alcohol.

$C_{22}H_{17}O_6N_3$. Calculated. C 62.98, H 4.10
Found. " 63.67, " 4.38

Because of the sparing solubility of this derivative it was used to obtain more of the hydrocarbon from accumulated mother liquors, followed by regeneration of the hydrocarbon with tin and HCl in acetic acid. This material was later used for oxidation experiments given below.

All the other more soluble crystalline hydrocarbon fractions were combined and converted into the picrate in alcoholic picric acid. This picrate, after recrystallization from the same solvent, gave what appeared to be a constant melting point of 125–126°. After decomposition of this picrate the regenerated hydrocarbon melted constantly at 124–125° after three recrystallizations from alcohol.

$C_{16}H_{14}$. Calculated. C 93.15, H 6.85
Found. " 92.71, " 7.06

The picrate prepared from the purified hydrocarbon formed rusty orange, flat needles or plates and melted now at 140–141° after recrystallization from alcoholic picric acid.

$C_{22}H_{17}O_7N_3$. Calculated. C 60.66, H 3.95
Found. " 61.00, " 3.77

The more insoluble high melting hydrocarbons from Fraction II were repeatedly recrystallized from alcohol, but no substance with constant melting point could be obtained.

From Fraction III, after several recrystallizations from alcohol and finally from butyl ether, a small amount of a hydrocarbon crystallizing as plates was recovered which melted constantly at 295–297°. In solution this substance exhibited a bright pale blue fluorescence.

$C_{21}H_{16}$. Calculated. C 93.99, H 6.01
Found. " 94.08, " 6.05
" 93.87, " 5.91

Quinone from the Hydrocarbon Melting at 127°—100 mg. of the hydrocarbon were dissolved in 5 cc. of acetic acid and heated on a steam bath for 1½ hours with 200 mg. of CrO_3 dissolved in 1 cc. of water. The mixture was carefully diluted, and after standing in the ice box the crystalline quinone was collected. After several recrystallizations from alcohol it formed brown-red prisms which melted at 203–205°.

With the small amount of this material on hand, its reaction with *o*-phenylenediamine in acetic acid solution was attempted. The crystalline quinoxaline formed cream-colored needles from chloroform alcohol which, after six recrystallizations, melted at 168–171°. Exhaustion of material prevented further recrystallization to possibly higher melting point.

Quinone from the Hydrocarbon Melting at 124–125°—100 mg. of the hydrocarbon were oxidized with CrO_3 as in the preceding case. The substance, after several recrystallizations from alcohol, formed garnet prisms which melted at 208–209°.

$C_{16}H_{12}O_2$.	Calculated.	C 81.32, H 5.12
	Found.	" 80.75, " 5.20

The quinoxaline prepared from this quinone after repeated recrystallization from chloroform alcohol melted constantly at 150°.

$C_{22}H_{16}N_2$.	Calculated.	C 85.69, H 5.23
	Found.	" 84.80, " 5.18

Dehydrogenation of Isostrophanthic Acid—50 gm. of α-isostrophanthic acid were heated in a dehydrogenation flask at 200° until evolution of CO_2 had ceased. The temperature was then raised to 320–340° during 6 hours, while selenium was added in 10 gm. quantities every hour until a total of 70 gm. had been added. The temperature was maintained at 320–340° for a further 30 hours. After extraction with ether the mixture was distilled at 0.2 mm. and the following cuts were taken: Fraction I, 185–210°, 13 gm., practically all crystalline; Fraction II, 210–240°, 7 gm., practically all crystalline. Both fractions were freed from possible oil by extraction of their picrates with petroleum ether and the regenerated hydrocarbons were fractionally recrystallized from alcohol according to the usual triangle scheme.

The most insoluble part of Fraction II melted constantly after several recrystallizations from alcohol at 140–142°. The substance crystallized as plates and exhibited a strong uniform lavender fluorescence under the fluorescent microscope.

$C_{16}H_{14}$. Calculated. C 93.15, H 6.85
Found. " 93.38, " 6.96
" 93.20, " 6.80
" 92.96, " 6.82

1.495 mg. substance; 30.535 mg. camphor; Δ 8.6°; mol. wt. = 228
2.820 " " 27.790 " " " 19.0° " " = 214
$C_{16}H_{14}$. Calculated. Mol. wt. = 206

The hydrocarbon formed a bright red picrate which apparently contained approximately 2 moles of picric acid for each mole of hydrocarbon. It proved to be too unstable to permit purification.

$C_{28}H_{20}O_{14}N_6$. Calculated. C 50.59, H 3.04
Found. " 53.88, " 3.10
" 53.76, " 3.42

The trinitrobenzene derivative, while unstable, could be recrystallized from alcoholic trinitrobenzene solution. It contained 2 moles of trinitrobenzene for each mole of hydrocarbon and formed bright orange plates which melted at 128–129°.

$C_{28}H_{20}O_{12}N_6$. Calculated. C 53.15, H 3.20
Found. " 52.96, " 3.44

From Fraction I no constant melting hydrocarbon could be obtained after more than 250 recrystallizations.

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STUDIES ON ORGANOGENESIS

I. THE ABILITY OF ISOLATED BLOOD CELLS TO FORM ORGANIZED VESSELS IN VITRO

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(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 13 TO 15

(Received for publication, June 21, 1934)

Blood cells, when placed for incubation in culture flasks containing a mixture of blood plasma and Tyrode solution, are capable of constructing highly organized, tubular processes that resemble, in some respects, the blood capillaries of the organism. It is the purpose of this communication both to describe these structures and to disclose the conditions under which they are formed.

Materials and Procedures

The cells are taken from blood that has been drawn from the carotid of adult chickens according to the procedures regularly employed in the preparation of plasma. The blood is collected in plain glass tubes and centrifuged immediately. No anticoagulant is used. After centrifugation, and the subsequent removal of the cell-free plasma yield, the thin layer of leukocytes (buffy coat) that rests above the red cells is coagulated by the addition of a drop or two of embryo tissue juice.[1] A little later, this layer can be removed as a disc and cut into small fragments for the preparation of the cultures. Each fragment consists of a layer of white cells, together with such red cells as remain adherent to them. After a brief period of irrigation with Tyrode solution, the fragments are placed in micro flasks[2] in a medium consisting of 0.2 cc. of adult chick plasma diluted with twice that amount of Tyrode solution. They are then placed for incubation at 37°C.

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[1] This material is rendered cell-free by freezing for two periods of 15 minutes each at −50°C.

[2] This flask (Carrel, A., *Compt. rend. Soc. biol.*, 1930, **105**, 826) is 30 mm. in diameter and 5 mm. in depth. It has a straight neck, 5 mm. in diameter. At the union of the neck with the chamber, there is a slight constriction which prevents the medium from running into the neck. The walls are of sufficient thinness (0.18–0.10 mm.) to permit the study of cultures with a 3 mm. oil immersion lens. At the termination of an experiment, the cultures may be fixed and stained ***in situ***.

EXPERIMENTS AND RESULTS

Early Stages in the Development of the Tubules.—As a rule, the tubules (Figs. 1–4) begin to be formed as soon as the cultures have been prepared. At one or more points on the margin of the implanted fragments (Fig. 4), a few red cells become dislodged and break away. Their places are taken by those from behind. If the proximal impact is great enough, these cells are also pushed out into the medium. This makes for a general streaming. Each cell that is forced out follows in the wake of those that have gone before. They may proceed in single file, or abreast of one another, but always over exactly the same route. This route may take any direction. It may become so branched as to form a system of great complexity (Fig. 1). But as long as the cells in the lead are being pushed forward by those from behind, they will continue to advance through the medium until it coagulates. The moment coagulation occurs, the pathway assumes the nature of a tunnel-like passage. Moreover, this passage will remain open and unobstructed, despite the fact that the cells in transit may be widely separated (Figs. 11 and 12). Its boundaries become the interphase between the fluid plasma within and the coagulated plasma without. By manipulating the flask, the cells that are enclosed can be made to flow in either direction. If coagulation occurs before the proximal outflow has ceased, its lumen may become densely packed with cells. Even after the medium has coagulated, however, the force of the outgoing cells may still be so great that a large spherical expansion is formed at its distal end (Fig. 3). At other times, the channels may be completely ruptured.

It has already been stated that the marginal outflow is initiated by the red cells. Red cells have smooth surfaces and easily shift their position. Without them, the phenomenon does not occur. Leukocytes, on the other hand, adhere to one another and to solid structures. While capable of independent locomotion, they may, as in the present instance, be swept along in a current of red cells. The outflow will not occur, however, if the buffy coat has become too firmly coagulated before the fragments have been prepared and placed in the flasks. Under these conditions, the red cells are held so securely in place by the fibrin meshwork of the explant itself that they are rendered incapable of movement. The greatest number of tubules are to be obtained from a thick fragment of relatively loose texture and one that contains an abundance of red cells.

It should also be pointed out that the tubule formation occurs independently of, and begins prior to, the general outward migration of leukocytes (Figs. 4 and 5). In fact, the two phenomena depend upon entirely different environmental conditions. It has been found, for example, that when the medium is composed of serum, the cells flow out irregularly from all sides, and no tubules are formed. In the presence of plasma, however, the outflowing cells may take every conceivable form, ranging from broad, fan-like disseminations through short, stalky, bud-like projections to long, slender ones such as those just described. It has also been found that the development of the tubules is not suppressed by substances that enhance cell activity unless they induce, at the same time, the immediate coagulation of the plasma. Thus, embryo tissue juice prevents their formation by producing immediate coagulation (Figs. 6 and 7). Their development is not hindered, however, by the addition of tryptic digests of fibrin. Although these latter substances stimulate the leukocytes to enormous activity, they do not hasten, to any appreciable degree, the coagulation of the fibrin network. It is, of course, possible to delay, to some extent, the onset of coagulation by retaining the cultures for a time at room temperature. This, as a rule, makes for the development of a larger number of tubules and for tubules of greater length than it is possible to obtain by immediate incubation.

Later Stages in the Development of the Tubules.—All that has occurred up to this point may take place within 15 to 20 minutes after the cultures have been prepared. As soon as the surrounding medium has become firmly coagulated, however, no further change occurs either in the length of the tube or in its diameter (Figs. 9 and 10). So far, in fact, the cultures have no definite walls. In the majority of cases, the walls begin to be laid down after from 5 to 8 hours' incubation (Fig. 8). They are formed by the activity of living cells, or cell products, particularly the thrombocytes, that have been deposited along the course of the tubules. Wherever there are gaps between the cells confined in the tubules, these minute bodies may be seen to spin out fine, hair-like filaments at the interphase between the lumen of the tubule and the surrounding coagulum (Figs. 11 and 12). These filaments are of the nature of fine mesenchymal fibrillae. Sometimes, they become very dense (Fig. 13). They take on a deep blue coloration when stained with Heidenhain's azan mixture, and become black when treated according to the Bielschowsky-Maresch silver impregnation procedures (Figs. 21 and 22).

Out in the surrounding medium, the thrombocytes may form similar strands, but here at random (Figs. 14 and 15). Where they are well isolated from one another, these strands are usually very short and are arranged radially (Fig. 14). The majority of them, however, tend to agglutinate. Very often, the agglutinated masses become joined together by numerous, parallel threads (Fig. 15).

After a few days, the red cells within the capillary-like formations become progressively phagocytized by the macrophages (Figs. 10 and 19). Eventually, these in turn may escape into the surrounding medium, sometimes through definite breaks in the walls, but more often by way of their proximal ends. After some days, the tube may be completely empty. In the meantime, however, any number of macrophages may come to rest on its outer surface (Figs. 16, 21 and 22). In this position, they bear striking resemblance to the much discussed cells of Rouget. Occasionally, their undulating membranes become so fused with one another as to give the impression of an unbroken syncytium. If the cultures were fixed and stained at this moment, the walls of the vessels would appear to be nucleated.

Sometimes the fibrous wall is incompletely formed, the strands being laid down over a single portion of the original passageway. At other times a single cord of fibrous material may extend along one side of it (Fig. 18). Invariably, however, its lumen remains open and filled with fluid. When the wall is complete, it is possible to inject the tubule with a micro pipette. With increasing pressure, the vessel becomes distended. Eventually, of course, it will burst.

DISCUSSION

The present experiments have shown that it is possible for the cells of the circulating blood, when placed for incubation in a suitable substratum, to provide themselves with organized vessels[8] of their own creation. In the beginning, the formation of the organized structures is a purely physicochemical phenomenon. It is dependent upon the consistency of the medium, the character and thickness of the original fragment and the surface peculiarities of the red cells. As soon as fragments containing a loose arrangement of red cells and leukocytes have been placed in a plasma mixture that does not coagulate immediately, certain of the red cells, as the result of physical pressure exerted upon them by neighboring cells, initiate a general outflow from the central mass into the surrounding medium. This leads to the formation of definite channels, or passageways, through the uncoagulated plasma. Eventually, the medium coagulates. Despite this, however, the plasma contained in the tubular channels remains fluid. Its properties have been altered, in some way, by the advancing blood cells. Then, as soon as the tubules have been formed, the thrombocytes that have been deposited at the interphase between the coagulated plasma without and the fluid medium within, proceed to lay down a fibrinated wall. They build this wall in exactly the same way that they produce at random short strands of fibrillae in the outer medium. Finally, the wall becomes covered by a membranous layer of leukocytes. Here again, the leukocytes manifest a common, well known property. They will cover a cotton fiber in quite the same manner (Fig. 17).

It follows, then, that the phenomenon as a whole is composed of a series of events that are both physicochemical and physiological in their nature. These various events are separate and distinct phenomena. They must, however, occur in a definite sequence. If, for example, the blood cells fail to stream out from the central explant before the medium coagulates, no tubules are formed. The streaming has been found to depend upon the physical features of the explant and the cells that comprise it; the rapidity with which the medium coagulates, on the other hand, depends upon the physicochemical nature of the various substances that go into it and also upon the temperature at which it is placed. Then again, the marginal outflow from the explant into the medium may carry only red cells. While this will insure the formation of the fluid channels, or passageways, through the plasma coagulum, the channels will not become endowed with fibrillar walls unless the red cells are accompanied by leukocytes and thrombocytes. Assuming, however, that the tubules have been formed and that they have developed fibrillar walls, the possibility of their being covered, eventually, by a cellular membrane depends entirely upon the nutritive quality of the medium.

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[8] Two years ago, Hueper and Russell (Hueper, W. C., and Russell, M. A., *Arch. exp. Zellforsch.*, 1932, **12**, 407) reported the appearance of similar structures in a certain percentage of "leucocyte" cultures, some of which had been prepared in much the same manner as those referred to above. The observations reported by these authors, however, are so completely at variance with those presented here that it does not seem advisable to attempt a comparison of the two accounts. Moreover, no pretence was made by them to determine the conditions under which the phenomenon occurred.

The formation of these structures is looked upon as a definite example of organogenetic development. The phenomenon takes place whenever a certain group of cells is placed in a certain type of medium. Given the proper set of environmental conditions, its occurrence is inevitable. It is true, of course, that the structures formed are only slightly analogous to the blood vessels formed in the organism. At the same time, their manner of formation has been such as to suggest that formative processes occurring in the organism might also depend upon just such an ordered sequence of physicochemical and physiological events.

SUMMARY AND CONCLUSIONS

1. Isolated blood cells, when placed for incubation in a plasma substratum, are capable of constructing highly organized, tubular processes that project from the explanted cell mass into the surrounding medium.
2. The tubular structures have fibrillar walls that may be covered, eventually, by a membranous layer of leukocytes. Their lumina contain blood cells suspended in fluid.
3. The formation of the tubules is initiated by the red cells. The leukocytes, and more particularly the thrombocytes, are responsible for the construction of the walls.
4. The phenomenon occurs only in the presence of plasma, the coagulation of which has been slightly delayed. Once the surrounding

medium has become firmly coagulated, no further change occurs either in the length of the tubules or in their diameter.

5. The development of the tubules is not suppressed by substances that enhance cellular activity unless they induce, at the same time, the immediate coagulation of the medium. The addition of embryo tissue juice prevents their formation by producing early coagulation.

6. The phenomenon as a whole is dependent upon the physicochemical nature of the medium, the character and thickness of the explanted fragment, and the physical and physiological peculiarities of the cells that comprise it. It is the expression of various physicochemical and physiological events that have occurred in a definite order, or sequence.

The author is greatly indebted to Mrs. Jane Bell Gladding, who has performed a large portion of the experimental work.

EXPLANATION OF PLATES

With the exception of those reproduced in Figs. 21 and 22, all of the photographs were made from living, unstained preparations. Eastman safety process films were used throughout.

PLATE 13

FIG. 1. Culture 5308-4. Tubule formations in a colony of blood cells photographed after 24 hours' incubation in plasma and Tyrode solution. The cells in the background are leukocytes that have migrated out from the same explant. ×31.

FIG. 2. Culture 5589-3. Portion of a tubule after 24 hours' incubation. The wall, clearly defined where it is present, is still incomplete. Note the miscellaneous character of the enclosed cells. ×198.

FIG. 3. Culture 4048-8. Tubule from a culture incubated for 30 hours. Note the spherical expansion at the distal end. ×99.

FIG. 4. Culture 4078-1. Tubule formations after 1 hour's incubation. The structures extend well beyond the zone of migrating leukocytes. ×28.

FIG. 5. Culture 4078-2. Tubule formations after 8 hours' incubation. By this time, the zone of outward cell migration extends well beyond the tubules. ×20.

FIG. 6. Culture 4092-10. Tubule formations in a colony of blood cells cultivated in plasma and Tyrode solution. As usual, no embryo tissue juice was added. Photographed after 5 hours' incubation. ×17.

FIG. 7. Culture 4092-3. Colony of blood cells cultivated in plasma, Tyrode solution and embryo tissue juice. In contrast to the culture shown in Fig. 6, no tubules were formed. Photographed after 5 hours' incubation. ×17.

FIG. 8. Culture 4048-7. Portion of a tubule after 6 hours' incubation. In this section of the tubule, a few red cells are to be seen. A spherical expansion at its distal end (top of figure) is not shown. ×164.

PLATE 14

FIG. 9. Culture 4048-7. The distal portion of a tubule photographed after 30 hours' incubation. The red cells contained in the distal expansion are evenly distributed. ×105.

FIG. 10. Culture 4048-7. The same as Fig. 9 after 3 days' incubation. By this time, the red cells have become phagocytized by the leukocytes. The several black blotches represent the location of phagocytes that are gorged with red cells. The proximal portions of the tubule are almost completely empty. ×105.

FIG. 11. Culture 4078-2. Portion of a tubule photographed after 8 hours' incubation. That section of the passageway lying between the two groups of enclosed red cells is open and unobstructed. Although the wall has not yet been formed, a single fibrous strand may be seen extending along the bottom of the tubule. ×365.

FIG. 12. Culture 4078-2. Portion of another tubule from the same culture after 8 hours' incubation. As in the case shown above, this tubule extends across the entire width of the photograph. The fibrous wall is in process of formation. Only those cells that are clearly in focus are contained within the tubule. ×365.

FIG. 13. Culture 4097-2. Portion of a tubule after 5 days' incubation. The fibrous wall is completely formed and is very dense. ×328.

FIG. 14. Culture 5016-3. Red cells, leukocytes and thrombocytes from the outer medium of a culture incubated for 24 hours. The thrombocytes have thrown out fine fibrillar strands that radiate in every direction. Just above the center of the figure a macrophage is seen clinging to one of the heavier connecting fibers. ×310.

FIG. 15. Culture 5016-3. Agglutinated masses of red cells, leukocytes and thrombocytes from another portion of the same culture after 24 hours' incubation. The various groups of cells are connected with one another by numerous fibrillae. ×310.

PLATE 15

FIG. 16. Culture 4048-8. Portion of a tubule after 5 days' incubation. Its surface is completely covered by leukocytes. Their thin, undulating membranes have become fused, giving the appearance of a syncytium. At the bottom and to the right of the figure, one of the cells lying on the surface of the tubule may be seen in relief. ×207.

FIG. 17. Culture 4087-10. Two sections of a single cotton fiber that has served as a supporting structure for free wandering leukocytes; from the outer medium of a culture of blood cells after 6 days' incubation. ×198.

FIG. 18. Culture 4048-9. Portion of a tubule whose cellular contents have

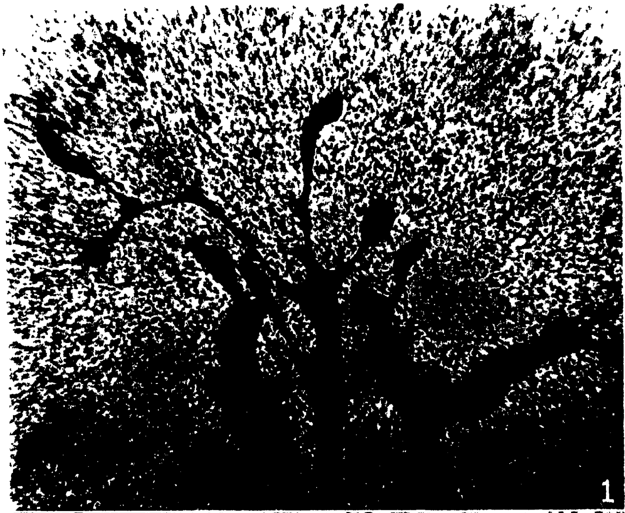
either died or migrated out from its lumen. Only a section of the tubule has been enclosed by a fibrous wall. Although the original channel branched out to the right (at the bottom of the figure), no wall was formed. Photographed after 8 days' incubation. ×185.

FIG. 19. Culture 4048-7. The distal portion of the same tubule shown in Figs. 9 and 10, after 8 days' incubation. ×185.

FIG. 20. Culture 4048-7. The distal portion of a tubule that has obtained a well formed, fibrous wall. The extreme tip of this tubule is of very small diameter. Photographed after 9 days' incubation. ×185.

FIG. 21. Culture 5925-3. Portion of a tubule from a culture fixed in Ringer-formol after 5 days' incubation and stained according to the silver impregnation procedures of Bielschowsky-Maresch. Note the complex, net-like structure of the fibrinated wall. ×422.

FIG. 22. Culture 5925-3. Another tubule from the same preparation shown in Fig. 21. A number of leukocytes have become incorporated in the fibrous wall. ×422.



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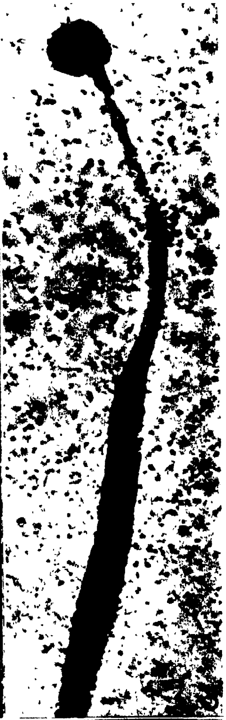

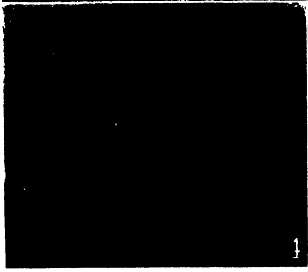

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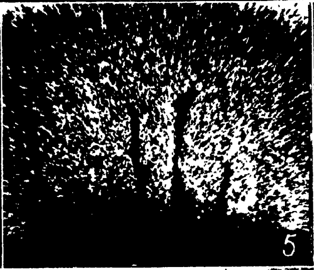

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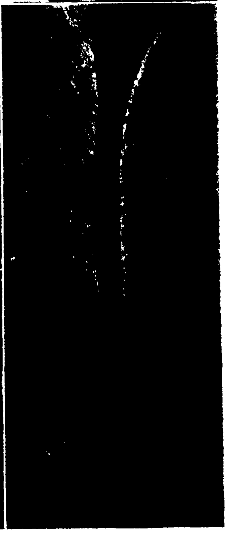

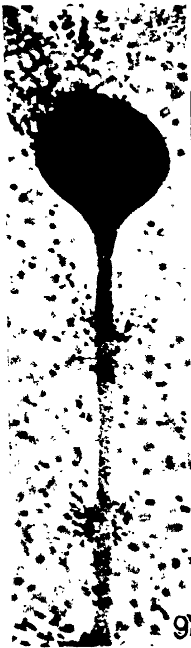
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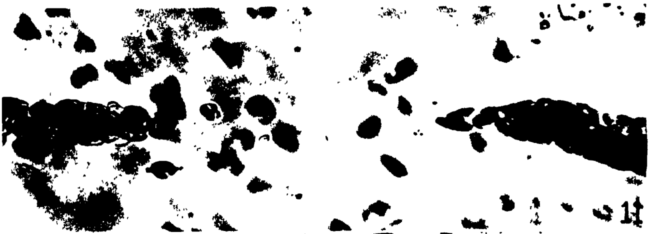
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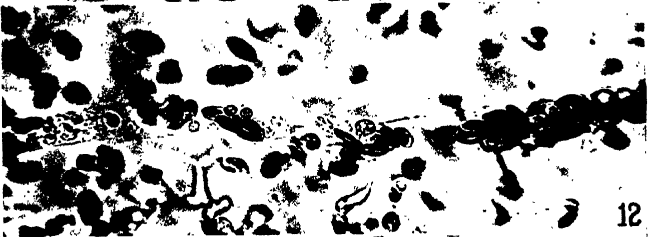
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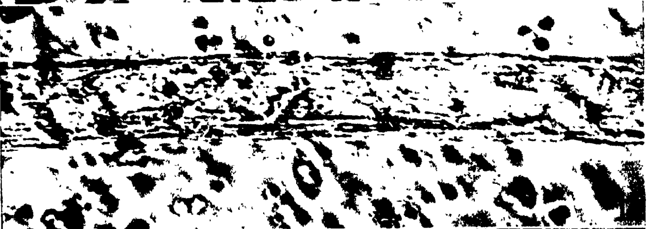

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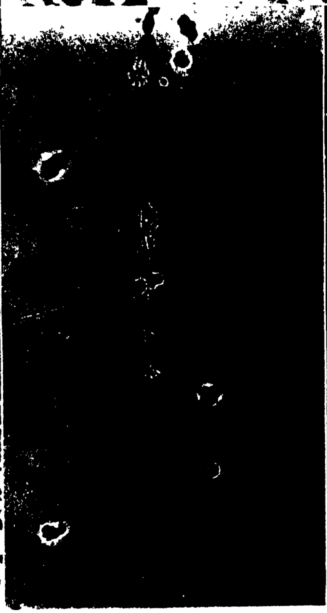

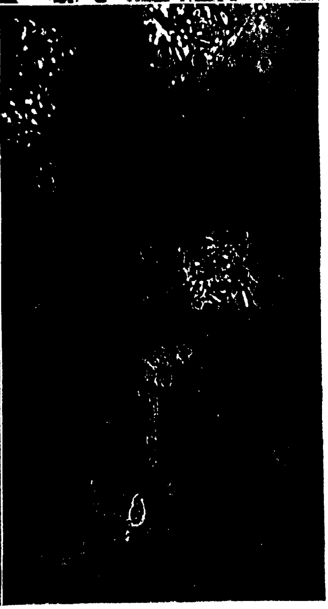

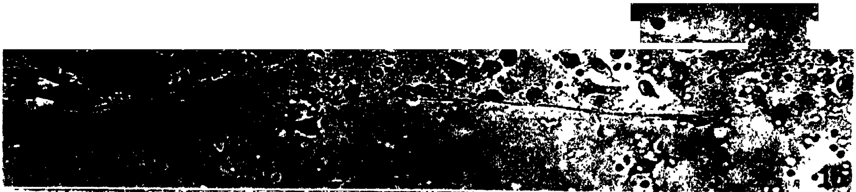

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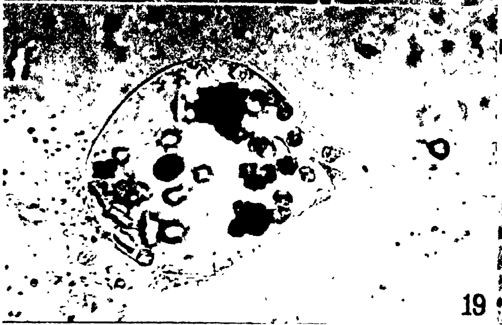

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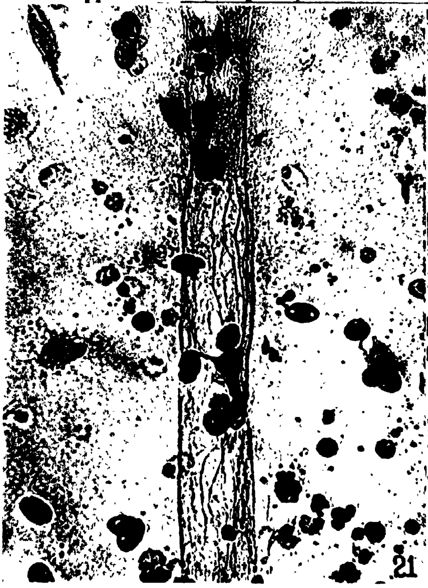

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(Parker: Studies on organogenesis. I)

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CRYSTALLINE ACETYL DERIVATIVES OF PEPSIN

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The isolation and identification of several individual enzymes has made it possible to attack the problem of their chemical structure. In the last few years it has been shown that urease (1), pepsin (2), trypsin (3), chymo-trypsin (4), probably pancreatic amylase (5) and rennin (24) are proteins, so that the general molecular structure of these enzymes is known. Enzymes have powerful catalytic properties, however, not possessed by ordinary proteins and therefore must possess characteristic chemical structures.

Since the chemical properties of a molecule are not the sum of the properties of its component parts, the catalytic activity of an enzyme must be considered as a property of the whole molecule and cannot, strictly, be referred to one special group. It is frequently true, however, that a particular property of the molecule is dependent upon a particular group or arrangement of the atoms within the molecule; the magnitude of this property depends upon the structure of the whole molecule. Thus organic acids usually contain a carboxyl group but the strength of the group depends on the entire molecular configuration. In the case of proteins possessing peculiar properties the first question to arise is whether the molecule consists entirely of amino acids or whether it contains a prosthetic group; *i.e.*, a compound not composed of amino acids. The peculiar properties of hemoglobin are ascribed to the presence of the prosthetic group, heme, which can easily be separated from the protein, globin. In this case the prosthetic group may be detected by its color. The yellow respiratory ferment of Warburg (6) is similar since again the prosthetic group is colored. In both these cases the characteristic properties are lost completely when the prosthetic group is separated from the rest of the molecule. The prosthetic group alone is therefore not responsible for the activity.

The products of hydrolysis of pepsin have no proteolytic activity and there is no other property known of the hypothetical group which in pepsin renders it active. Indirect information as to the nature of the active group may be obtained, however, by altering the structure of the original pepsin molecule with reagents known to react with certain chemical groups and determining the effect of this change in structure upon the activity. By analogy with other compounds, such as acids or dyes, it would be expected that any change in the molecule would affect the activity but that the extent of the effect would depend upon a number of factors such as the nature of the reagents, the reacting groups of the molecule, and their relation to the active part of the molecule. Pepsin is known to have such free groups as carboxyl (COOH), amino (NH_2), and phenolic hydroxyl (OH). The effect on the activity resulting from the modification of these groups should furnish information as to their relation to the molecular structure responsible for the activity. If the modification of these groups in native pepsin resulted in a complete loss in activity it would be probable that the active group, or one closely associated with the active group, had been altered by the treatment. If the modification of these groups failed to affect the activity it would follow that the modified groups are not closely related to the active part of the enzyme. The present experiments were designed to determine what effect acetylation of the various groups of pepsin would have on the activity.

The action of a number of biological substances is markedly altered by the introduction of one or more acetyl groups into the molecule. Hunt (7) has shown that acetyl choline has at least 40,000 times as much action on blood pressure as does choline. Warburg and Negelein (8) have prepared a diacetyl deutero hemoglobin which has only 52 per cent of the capacity to combine reversibly with oxygen as does ordinary hemoglobin. Workers in several laboratories have studied the change in properties of crystalline insulin after acetylation. Freudenberg and his coworkers (9) have reported complete inactivation of crystalline insulin by acetylation. Part of the activity could be recovered by treatment of the inactive acetylated insulin with dilute alkali. Later, using freshly precipitated amorphous insulin, they reported a product of acetylation which had 5–10 per cent of the original activity. The amino nitrogen was reduced from 1 per cent to 0.1 per cent by the acetylation. More recently Avery and Goebel (10) have reported that an acetyl group present in the Type I specific pneumococcus polysaccharide renders the material antigenic. This property disappears on removal of this one acetyl group.

Hugounenq and Loiseleur (11) have reported that treatment of crude pepsin solutions with methylating and diazotizing agents failed to affect the proteolytic activity of the enzyme. Their treatment was directed toward the primary amino groups and since they noticed no change in activity of the enzyme they concluded that the primary amino group had little if anything to do with the proteolytic activity. They did not, however, measure either the loss in amino groups nor increase in methyl groups, nor did they isolate any compounds.

In the present experiments pepsin was acetylated by treatment with ketene and it was found that the activity decreased as the acetylation proceeded. In the study of the course of the acetylation reaction it became apparent that the time rate of change in the specific activity of the enzyme during acetylation depends upon the pH of the solution. Acetylation of pepsin at pH 5.5 causes a greater drop in specific activity per acetyl group introduced than acetylation at pH 4.5. If the pH of the pepsin solution is kept constant the specific activity of the reaction mixture when plotted against the increase in number of acetyl groups per molecule of protein drops off logarithmically as acetyl groups are introduced. There is no sharp break in the curve; the activity drops gradually and is 10 per cent or less of the original activity after the introduction of more than twenty acetyl groups per molecule of enzyme.

A preparation of acetylated pepsin has been obtained in crystalline form which has 60 per cent of the activity of the original pepsin, as measured by several methods. This material contains one or less primary amino groups per molecule of protein instead of the three or four originally present in pepsin. It also possesses from six to eleven acetyl groups per molecule, the number depending upon the conditions during the acetylation. From the acetyl determinations of several different preparations and the solubility experiments it appears that there may be several acetyl products possessing approximately 60 per cent of the original pepsin activity and which may form solid solutions with one another.

Another crystalline acetylated pepsin preparation was obtained by subjecting the above acetyl pepsin to normal sulfuric acid. The specific activity of this acid solution increased to the value of pure pepsin and the acetyl content dropped from nine to four acetyl groups per molecule. There was no measurable increase in the primary amino nitrogen and it is probable that the remaining acetyl groups are on the primary amino groups. It was also possible to prepare this same, or a closely related material, starting with pepsin. By careful acetylation an acetyl product was obtained which had lost none of its activity but which had lost all of its primary amino groups. This material had three acetyl groups per molecule, the equivalent of the amino groups lost.

Still another acetylated product was prepared by acetylation for 35–40 hours at pH 5.5 in a dialyzing bag. This material was only 10–15 per cent as active as pepsin and had on the average twenty acetyl groups per molecule. On further acetylation the decrease in activity was very slow. It was much less soluble than the other preparations and crystallized with difficulty and in very small crystals.

The different acetyl preparations could be separated from one another by fractional precipitation from concentrated magnesium sulfate solution. Under the microscope the crystals of all of the acetyl preparations appeared alike and indistinguishable from those of pepsin as may be seen in Fig. 1.

The results of these acetylation experiments show that acetylation of primary amino groups does not change the specific activity of pepsin by more than 10 per cent but that addition of acetyl groups in some other places causes a marked decrease in activity.

The primary amino groups of proteins in general are those of lysine and since acetylating these groups has little or no effect on the activity it is probable that the lysine groups are not part of or are not intimately associated with the molecular structure responsible for the activity. Further acetylation, however, causes a marked decrease in activity which would indicate that certain groups of the protein more closely related to the active part of the molecule have been acetylated. Ketene reacts with NH, OH, or COOH groups and it is not possible to decide at present between the various possibilities. The ease with which these groups may be removed suggests that OH, or COOH groups have been acetylated.

The pH activity curves, acid and alkali inactivation, and the titration curves of the acetyl preparations examined were not greatly different from the same properties of the parent substance, pepsin. On the other hand no isoelectric point could be detected for the "100

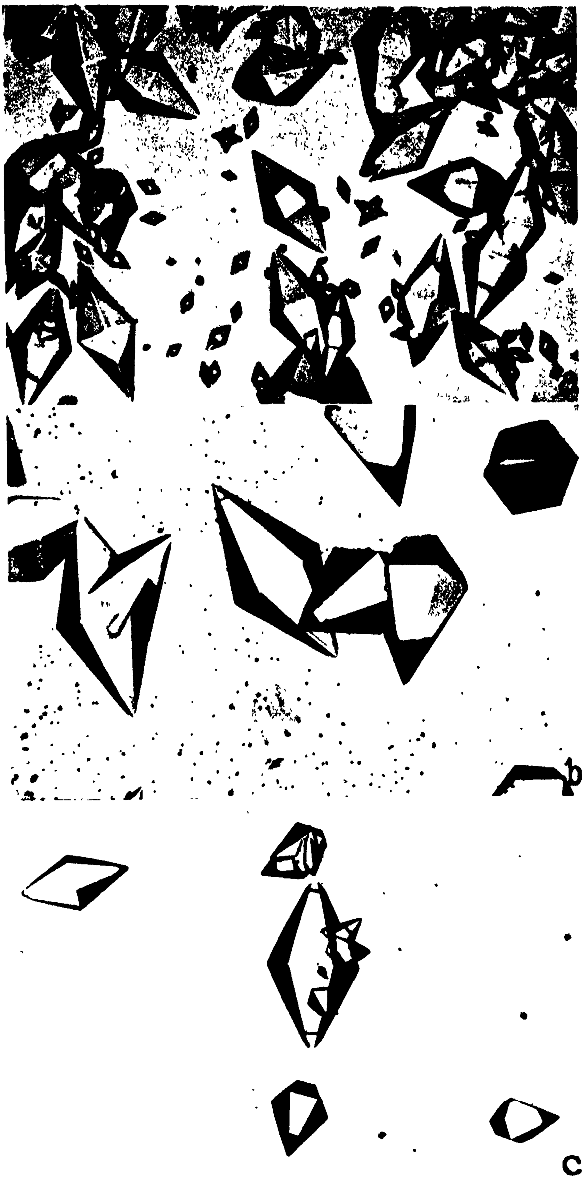


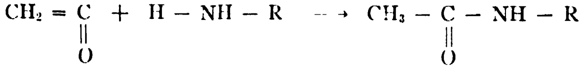
FIG. 1. (*a*) Crystalline pepsin. (*b*) Crystalline "100 per cent active" acetyl pepsin. (*c*) Crystalline "60 per cent active" acetyl pepsin.

per cent active" and "60 per cent active" acetylated derivatives and their solubilities in acid decreased as the concentration of acid increased. These two properties are quite different from those of pepsin which has an isoelectric point at pH 2.7 and the solubility of which increases as the solution is made more acid than pH 2.7.

EXPERIMENTAL RESULTS

Acetylation with Ketene

Staudinger (12) in his study of ketene ($CH_2 = C = O$) pointed out that ketene is capable of reacting in dilute acid but the reaction is more rapid in alkaline solution and that whereas it reacts with all of the following groups—primary and secondary amines, aliphatic alcohols, water and phenols—the rates of reaction are quite different for each group. Primary amines react most rapidly and the phenols least rapidly. The reactions of ketene are in general addition reactions and polymerizations, the latter being of little or no consequence in this work. The addition reactions may be illustrated as follows:



$$CH_2 = \underset{\underset{O}{\|}}{C} + H - NH - R \rightarrow CH_3 - \underset{\underset{O}{\|}}{C} - NH - R$$

Acetylation of amino acids using ketene has been reported by Bergmann and Stern (13). This method is more suitable for the acetylation of pepsin than the usual methods since the reaction may be carried out in slightly acid aqueous solution and without any appreciable denaturation of the protein.

The ketene used in this work was generated by the thermal decomposition of acetone vapor (14).

Course of the Acetylation Reaction

Effect of pH.—In the early experiments it was noticed that when ketene was passed into a 5–10 per cent pepsin solution in 3 M pH 5.5 acetate buffer, the specific activity of the enzyme dropped rapidly at first and then more slowly as the reaction progressed. When the specific activity reached approximately one-half that of the original pepsin there was no further appreciable change with continued exposure to ketene. It was first thought that this flattening out of the specific activity-time curve indicated the completion of the acetylation reaction and a product of definite properties different from those of pepsin was repeatedly obtained. The pH, however, was not held constant. The acidity of the enzyme solution changed from pH 5.5 to 4.0 during the course of the acetylation experiment. This might be expected for, as previously pointed out, ketene reacts with water to form acetic acid.

When the acetylated product described above, with a specific activity of 60 per cent that of pepsin, was precipitated out of the pH 4.0 buffer and dissolved in pH 5.5 acetate buffer and ketene run in as before, the specific activity again dropped and again reached a nearly constant value as the pH of the solution approached pH 4.0. This product was precipitated and redissolved in fresh pH 5.5 buffer and as before subjected to ketene and again there was an initial drop in activity followed by a slowing up of the time rate as the pH of the solution dropped toward pH 4.0.

Experiment I—Course of Acetylation as pH Varied from pH 6.0–4.0.—175 gm. of twice crystallized Cudahy pepsin filter cake was dissolved in 3 M sodium acetate; final volume was 400 ml. Ketene was run in with stirring. 20 ml. samples were withdrawn at various intervals of time and the following analyses made using the methods described in the section devoted to experimental methods—pH, protein nitrogen, non-protein nitrogen, Van Slyke amino nitrogen, proteolytic activity by the hemoglobin method, and acetyl content. After 800 minutes exposure to ketene the protein was precipitated out by the addition of an equal volume of saturated magnesium sulfate. The precipitate was filtered off, dissolved in 3 M sodium acetate, and the final volume made up with sodium acetate to the volume of the solution just prior to precipitation. Ketene was passed in as before for 900 minutes or a total of 1700 minutes. The protein was again precipitated as before and dissolved in fresh sodium acetate and ketene run in for 300 minutes or a total of 2,000 minutes. The analyses of the samples are plotted in Curve I of Fig. 2; Curve I of Fig. 3; and in Fig. 5.

The results of this series of experiments may be seen graphically in Curve I (Fig. 2). They indicate that the change in slope of the time-specific activity curve was due to a change in the pH of the solution and not to the completion of the acetylation. It was later found that the pH could be kept nearly constant by placing the buffered pepsin solution in a collodion bag suspended in a large volume of buffer. As a check experiment, therefore, acetylation was carried on in a collodion dialyzing bag with the pH nearly constant between pH 5.0 and 6.0. Under these conditions the drop in specific activity of the reaction mixture with time did not show a decided change as the specific activity approached 60 per cent of the original pepsin. As may be seen from Curve II of Fig. 2 the activity continued to drop with only a very gradual change in the slope of the curve and seems to show that the differences noted in the acetylation of pepsin are differences in time rates and are probably caused by the differences noted in the pH values of the solutions. However, when the increase in acetyl groups was plotted against specific activity (see Fig. 3) the points obtained from acetylation at pH 4.5 and at pH 5.5 did not fall along the same curve.

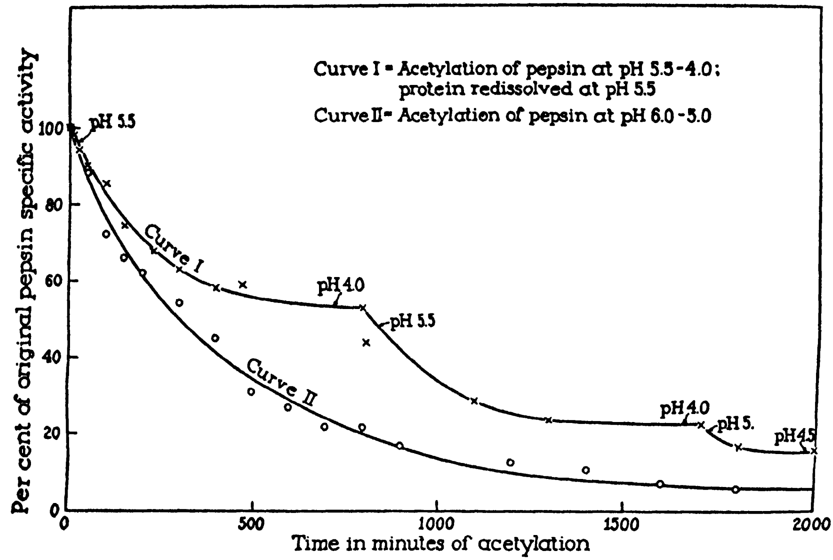


Fig. 2. Per cent specific activity of pepsin during course of its acetylation

Experiment II—Course of Acetylation at Constant pH 5.5.—135 gm. of once crystallized pepsin, prepared from Cudahy pepsin was twice precipitated by half saturated magnesium sulfate at pH 4.0 and dissolved in 2 M acetate buffer pH 5.8 so that the final volume was 400 ml. The enzyme solution was placed in a collodion bag prepared from Merck's U. S. P. collodion and this set into 2 liters of the same buffer as that used for the solution of the protein. Ketene was run in rapidly and the solution stirred both inside and outside of the bag. At the end of the day the bag and solutions were placed in the ice box and the reaction started again the next morning. 20 ml. samples were withdrawn and analyzed for protein, non-protein nitrogen, Van Slyke amino nitrogen, pH, activity by the hemoglobin method, and acetyl content. The results of the analyses are to be found plotted in Curve II of Figs. 2 and 3; Curve I of Fig. 4; and in Fig. 5.

These curves are independent of time and should, therefore, be collinear if the time rate is the only factor in the acetylation of pepsin affected by a change in pH. There was a much greater drop in specific activity per unit increase in acetyl groups when the acetylation was carried on at pH 5.5 than at pH 4.5. Curves I and II of Fig. 3 were plotted from data of the same experiments as were the Curves I and II of Fig. 2. Curve III of Fig. 3 represents an experiment in which the pepsin was acetylated at pH 4.5–4.0.

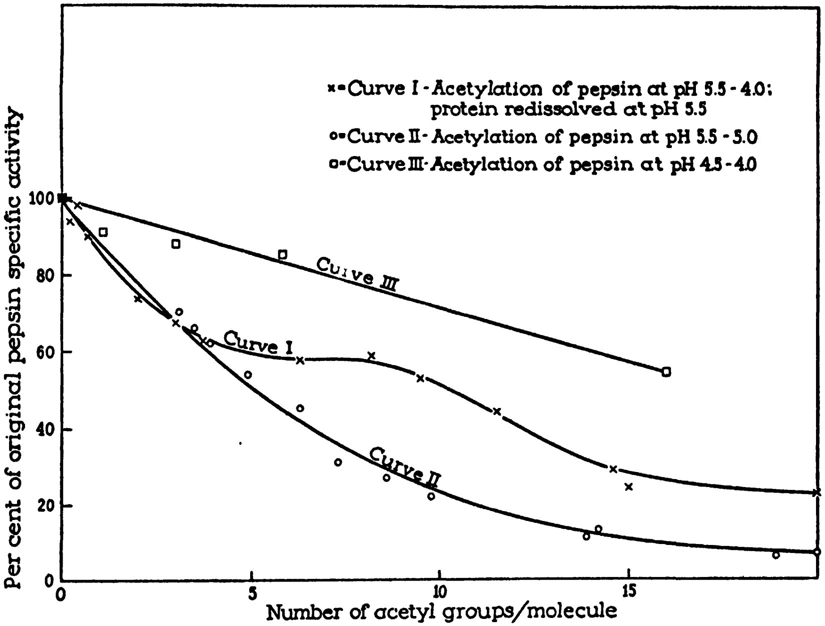


FIG. 3. Effect of a change in acetyl groups per molecule on specific activity of pepsin.

Curves I and II of Fig. 4 are the data of Curves II and III of Fig. 3 plotted on logarithmic scale. It may be seen from Fig. 4 that when the pH is constant the change in specific activity of pepsin during acetylation varies logarithmically with the increase in acetyl groups per molecule.

Experiment III—Course of Acetylation at Constant pH 4.5.—45 gm. of twice crystallized Cudahy pepsin filter cake washed four times with half saturated magnesium sulfate was dissolved in 6 M pH 4.5 acetate buffer so that the final volume was 150 ml. Ketene was passed in rapidly with stirring. 20 ml. samples were withdrawn and analyzed for protein, non-protein, and amino nitrogen, activity by the hemoglobin method, pH, and acetyl content.

The results are plotted in Curve III of Fig. 3; Curve II of Fig. 4; and in Fig. 5.

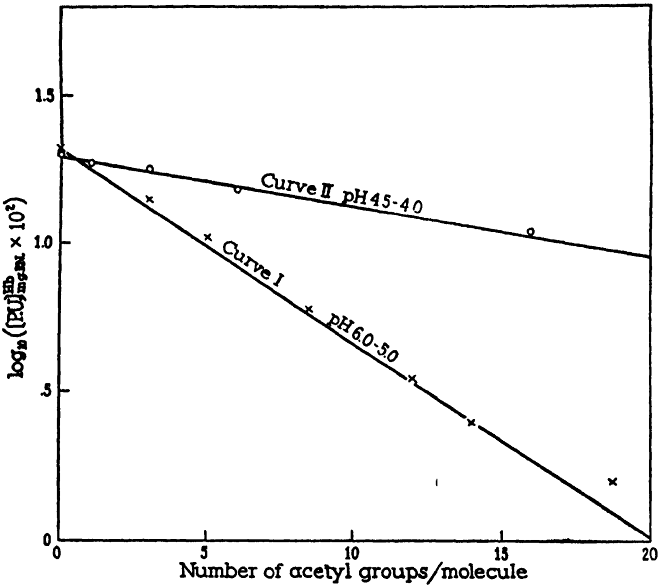


FIG. 4. Logarithm of specific activity of pepsin as affected by a change in the acetyl groups per molecule, acetylation at nearly constant pH.

It may be seen that there was only one-third the drop in specific activity per unit increase in acetyl groups at pH 4.5 as when the enzyme was acetylated at pH 5.5. Evidently acetylation occurs at different groups, depending upon the pH. This is probably due to the effect of ionization upon the rate of acetylation of the various groups present in the original pepsin.

Change in Primary Amino Groups during Acetylation.—The change in free primary amino groups was also followed during the course of the acetylation reaction by means of Van Slyke's micro amino nitrogen method (15).

Pepsin contains an unusually small number of free amino groups, 0.15 per cent or three to four groups per molecule[1] calculated from a molecular weight of 36,000. Most proteins contain between 0.75–1.5 per cent amino nitrogen or twenty to forty free amino groups per molecule. Fig. 5 contains the curves obtained by plotting the amino nitrogen content against specific activity as acetylation proceeds at pH 5.0–6.0 and pH 4.0–4.5. There seems to be no difference in the loss in activity compared to the decrease in amino nitrogen whether the acetylation is carried out at pH 4.5 or pH 5.5. The results show that there is not more than a 20 per cent drop in specific activity with a loss of practically all of the amino nitrogen. Measurements of less than one amino group per molecule were unreliable.

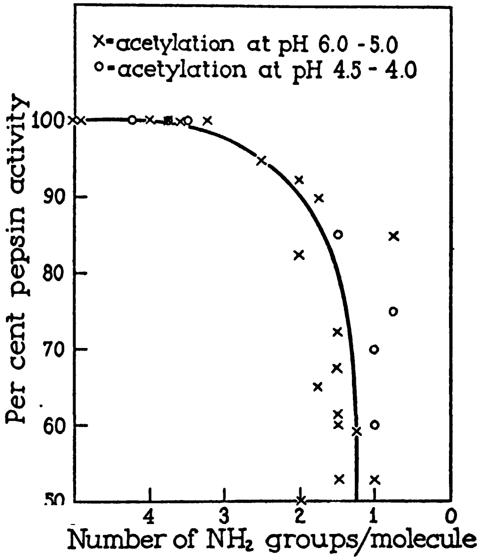


FIG. 5. Effect of acetylation of free amino groups on specific activity of pepsin.

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[1] This is lower than the value first reported (2), the difference probably being due to the presence of non-protein nitrogen in the pepsin preparation of the earlier analysis.

Preparation and Crystallization of the Acetyl Derivatives

Three preparations of acetylated pepsin have been obtained which differ in specific activities, number of acetyl groups per molecule, and in other properties. For convenience they have been named according to their specific activity relative to that of pepsin and spoken of as though they were pure chemical individuals although in fact they may be solid solutions of closely related compounds.

TABLE I

Preparations of Acetylated Pepsin

Preparation	Method of preparation	Specific activity $[P.U.]^{Hb}_{mg.\,P.N.}$	Pepsin specific activity	Amino nitrogen	No. of amino groups per molecule	Acetyl	No. of acetyl groups per molecule
			per cent	*per cent*		*per cent*	
Pepsin		0.22		0.16	4.0	0.12	1
"100 per cent active" acetyl pepsin	Acetylation of pepsin at pH 5.5 for 45 min.	0.21	100	0.02	0.5	0.51	4
"100 per cent active" acetyl pepsin	Hydrolysis of "60 per cent active" acetyl pepsin in N/1 sulfuric acid at 8°C. for 67 hrs.	0.20	100	0.02	0.5	0.44	4
"60 per cent active" acetyl pepsin	Acetylation of pepsin at pH 5.5-4.0 for 12 hrs.	0.12	55	0.04	1.0	1.0	8
"10 per cent active" acetyl pepsin	Acetylation of pepsin at pH 5.5 for 35 hrs.	0.03	14			2.7	23

Experimental Procedure

"100 Per Cent Active" Acetyl Pepsin.—This material was prepared by dissolving 100 gm. of twice crystallized pepsin filter cake containing less than 10 per cent of the total nitrogen as non-protein nitrogen, in 300 ml. of 3 M sodium acetate and then passing ketene into this solution slowly for 50–75 minutes. One bubble of gas every second from an orifice 25 mm. in diameter was found a satisfactory rate. After 50–75 minutes analysis of protein, non-protein, and amino nitrogen, activity, and acetyl content showed a loss of not more than 10 per cent of the activity but a loss of all measurable amino nitrogen. The protein was precipitated from solution by the addition of an equal volume of saturated magnesium sulfate after first adding acid to make the solution approximately pH 4.5. This precipitate was dissolved in water, precipitated by titrating to pH 3.0 with acid, and the precipitate

dissolved in water with the aid of sodium acetate and crystallized after the procedure of Northrop (2).

"60 Per Cent Active" Acetyl Pepsin.—125 gm. of once crystallized pepsin filter cake was dissolved in 3 M sodium acetate. The final volume was 400 ml. Ketene was passed in rapidly for 12–14 hours. Analyses for protein, non-protein, and amino nitrogen, activity, pH, and acetyl content were made. The solution was then subjected to the same treatment as the preceding solution; the protein precipitated with magnesium sulfate, acid, and thence crystallized.

"10 Per Cent Active" Acetyl Pepsin.—125 gm. of twice crystallized Cudahy pepsin filter cake was dissolved in saturated sodium acetate solution so that the final volume was 400 ml. This solution was placed in a collodion bag of 500 ml. volume. The collodion bag containing the enzyme solution was placed in 2 liters of 3 M sodium acetate and ketene passed in for 35 hours. The solution was filtered, and the protein precipitated and crystallized as above.

Analytical results found in Table I are typical of several preparations of each product. The derivatives were crystallized and were, in general, more stable than pepsin, possibly because they were less soluble than pepsin.

The experimental procedure for the preparation of "100 per cent active" acetyl pepsin by acid hydrolysis of the "60 per cent active" acetyl pepsin is discussed on page 248.

Evidence Concerning the Purity of the Crystalline Acetyl Derivatives of Pepsin

Fractionation Experiments.—No evidence could be found for the existence of an acetyl derivative whose specific activity was between 100 per cent and 60 per cent of the activity of pepsin. This follows from the results of the fractionation experiment summarized in Table II. A large portion of a reaction mixture, the specific activity of which had been reduced to 75 per cent of its original value by acetylation with ketene, could be fractionated into two parts possessing approximately 60 per cent and 100 per cent of the specific activity of pepsin. Further fractionation failed to demonstrate any protein fraction possessing a lower specific activity.

It might be supposed that the "60 per cent active" acetyl pepsin was not a pure compound but a mixture or solid solution of pepsin and an inactive or weakly active acetyl derivative. Table III contains the results of some experiments designed to test this question.

The results in Part A are typical of several fractionation experiments on a number of the ten to fifteen preparations of "60 per cent active" acetyl pepsin. The results in Part A show that the "60 per cent active" acetyl pepsin, as ordinarily prepared, could not be fractionated into pepsin and a protein having a lower specific activity. A protein having an activity corresponding to that of pepsin was separated but the amount was insignificant though this amount of the material brings out the sensitivity of the fractionation experiments in detecting the presence of even closely related substances. Part B of Table III reveals that an equimolecular mixture of pepsin and "10 per cent active" acetyl pepsin which resembled very closely the "60 per cent active" acetyl pepsin could be fractionated with comparative ease into two fractions having higher and lower activities than the mixture. It seems probable, therefore, that the "60 per cent active" acetyl pepsin prepared according to the directions in the previous section is not a mixture or solid solution of the original pepsin with a derivative of very low activity.

TABLE II

Fractionation of a Reaction Mixture Possessing 75 Per Cent of the Original Activity

			N/ml.		$[\text{P. U.}]^{\text{Hb}}$			
	No.	Volume	Total	Protein	1/ml.	1/mg. P.N.	Total P.N.	Total P.N.
		ml.	*mg.*	*mg.*			*mg.*	*per cent*
No. 4 of Dec. 28, 1933, reaction mixture pH 6.0	1	20	14.2	13.1	2.1	0.16		
No. 1 + 5 ml. of 5 N sulfuric acid, stirred till precipitate dissolved; pH 4.5	2	25	11.4	10.5	1.7	0.16	263	100
No. 2 + 7 ml. saturated magnesium sulfate; filtered	3							
Residue No. 3 dissolved in water	4	10	8.1	7.75	1.0	0.13	77.5	30
Filtrate No. 3 + 9 ml. saturated magnesium sulfate; filtered; filtrate	5	37	0.63	0.20	0.05	0.25	7.4	3
No. 4 after samples had been removed	6	8	8.1	7.75	1.0	0.13	62.0	100
No. 6 + 4 ml. saturated magnesium sulfate; centrifuge; decant	7							
Residue No. 7 + water	8	6		2.5	0.34	0.136	15.0	24

TABLE III

Fractionation of "60 Per Cent Active" Acetyl Pepsin and of a Mixture of Pepsin and 10 Per Cent Active Acetyl Pepsin

	No.	Volume	N/ml.		[P. U.]^{Hb}		Total P.N.	Total P.N.
			Total	Protein	1/ml.	1/mg. P.N.		
			mg.	*mg.*			*mg.*	*per cent*
Enzyme Solutions								
2 X crystallized Cudahy pepsin Nov. 1, 1933, dissolved in water + sodium acetate; concentration adjusted with water	1		5.65	5.50	1.13	0.21		
No. 10 of Sept. 25, 1933, "10 per cent active" acetyl pepsin + saturated magnesium sulfate, filtered; residue dissolved in water + sodium acetate	2		6.3	5.50	0.20	0.036		
No. 5 of October 23, 1933, "60 per cent active" acetyl pepsin	3		11.3	10.30	1.30	0.126		
Part A. Fractionation of "60 Per Cent Active" Acetyl Pepsin								
No. 3 + acid to pH 4.5; + equal volume saturated magnesium sulfate; filter; residue dissolved in M/10 pH 4.65 acetate buffer	4	75	7.14	6.9	0.84	0.122	520	100
No. 4 + 22 ml. saturated magnesium sulfate; stirred; centrifuge; decant	5							
Residue No. 5 + water	6	13	11.1	10.6	1.3	0.123	138	27
Supernatant No. 5 + 40 ml. saturated magnesium sulfate; stirred; (test showed practically no protein in solution) + 20 ml. M/10 pH 4.65 acetate buffer; stirred 10 minutes and filtered; filtrate	7	160	0.203	0.12	0.026	0.22	19	4
Part B. Fractionation of an Equimolecular Mixture of Pepsin and "10 Per Cent Active" Acetyl Pepsin								
10 ml. No. 1 + 10 ml. No. 2 (crystals formed; 4 ml. 4 N sodium acetate added to dissolve these crystals; to neutralize the sodium acetate 3 ml. of 5 N sulfuric acid was added; 1 ml. removed for analysis)	8	26		4.1	0.50	0.12	107	100
No. 8 + 1.2 ml. saturated magnesium sulfate with stirring; centrifuge; decant	9							
Residue No. 9 dissolved in water	10	5		4.55	0.31	0.068	23	21
Supernatant No. 9 + 10 ml. saturated magnesium sulfate; centrifuge; supernatant	11	40	0.69	0.36	0.088	0.24	14	13

Solubility Experiments

Experimental Procedure.—The methods used in these solubility determinations were identical with those described by Northrop (16).

The solubility of a pure substance is independent of the quantity of solid phase. Northrop (2) found that in all the solvents used pepsin solubilities agree with this rule and the results indicate, therefore, that pepsin is a pure substance.

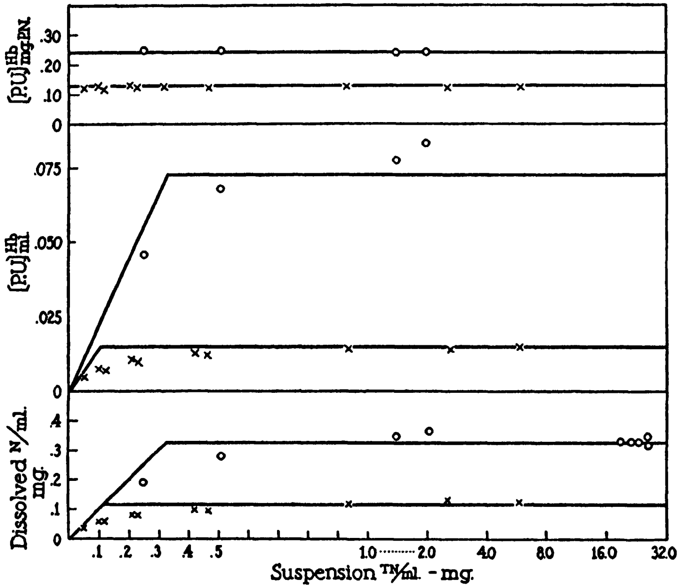


FIG. 6. Solubility of amorphous "100 per cent active" acetyl pepsin and of amorphous "60 per cent active" acetyl pepsin in 0.05 M pH 4.65 acetate buffer and 0.5 saturated magnesium sulfate.

The solubilities of two of the acetylated pepsin preparations have been determined. The results of these experiments are plotted in Fig. 6. They are also summarized with the pepsin solubilities in Table IV. The results show definitely that the specific activity, *i.e.* ratio of activity to protein nitrogen, is constant and independent of the quantity of solid phase present. This confirms the results of the fractionation experiments. The points obtained in the region of a small quantity of solid material for the "60 per cent active" acetyl pepsin do not fall on the theoretical curve. Although the figures are small and consequently subject to a greater percentage error, the writers believe that the results are significant in demonstrating that the material, as prepared, is a solid solution of two or more substances having nearly the same specific proteolytic activity.

Solubility of Acetylated Pepsin in the Presence of Pepsin

Experimental Procedure.—The suspensions of "60 per cent active" acetyl pepsin and of pepsin used in the solubility experiments of the previous section were used in the present experiments. The solvent was also the same (0.5 saturated magnesium sulfate—0.05 M pH 4.65 acetate buffer). The two suspensions were mixed in varying quantities, keeping the final volume constant. They were then dissolved in the acetate buffer part of the solvent and precipitated by stirring in the saturated magnesium sulfate just as in the solubility experiments. In every case there was an excess of solid of both constituents after precipitation. They were then filtered and the filtrates analyzed for activity and nitrogen.

TABLE IV

Solubilities of Pepsin and Acetyl Derivatives

Solvent—0.5 saturated magnesium sulfate sp. gr. 1.295
0.05 M pH 4.65 acetate buffer
Temperature = 25°C. ± 2°C.

Material	N/ml.	
	Amorphous	Crystalline
	mg.	*mg.*
Pepsin (Northrop)	1.0	0.22
Pepsin (Herriott)	1.1	
"100 per cent active" acetyl pepsin	0.33	
" 60 per cent active" acetyl pepsin	0.12	0.06

The results of this experiment are shown in Fig. 7. They bring out quite clearly that the "60 per cent active" acetyl pepsin forms solid solutions with pepsin.

Constancy of Properties during Fractional Crystallization

Table V contains the analyses of seven fractional crystallizations of a preparation of "60 per cent active" acetyl pepsin. In the course of seven recrystallizations there is a loss of 80 per cent of the original material but no drift in any of the properties measured.

Increase in Specific Activity on Removal of Acetyl Groups by Acid Hydrolysis

Since pepsin is instantly denatured in alkaline solution preliminary studies of the hydrolysis of acetyl groups from acetylated pepsin were carried out in acid solutions. At room temperature and with an acid concentration in which the hydrolysis of acetyl groups was appreciable there was too great a loss of enzyme through inactivation. By

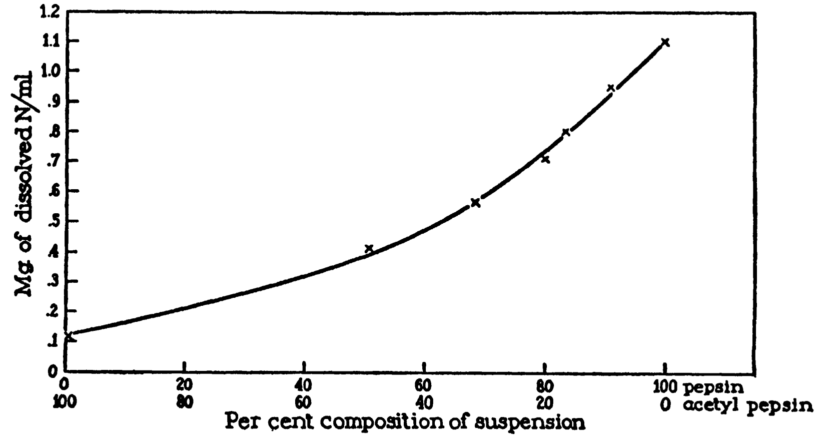


FIG. 7. Solubility of mixtures of amorphous pepsin and amorphous "60 per cent active" acetyl pepsin in 0.05 M pH 4.65 acetate buffer and 0.5 saturated magnesium sulfate.

TABLE V

Constancy of Properties of "60 Per Cent Active" Acetyl Pepsin during Fractional Crystallization

Crystallization No.	Quantity of crystals	$[\text{P. U.}]^{\text{Hb}}_{\text{mg. P.N.}}$	$[\alpha]^{24°\text{C.}}_{\text{D}}$ pH 4.7
	gm.		
1	62	0.125	83
2	40	0.129	92
3	36	0.123	84
4	30	0.125	94
5	20	0.118	85
6	15	0.126	84
7	10	0.120	86

TABLE VI

Increase in Specific Activity on Removal of Acetyl Groups by Acid Hydrolysis

Procedure	No.	Time in acid	Volume	N/ml.		$[P.U.]^{Hb}$		NH_2-N No./mol.	Acetyl No./mol.
				Protein	Non-protein	1/ml.	1/mg. P.N.		
		hrs.	*ml.*	*mg.*	*mg.*				
Acetylated pepsin No. 15 of July 11, 1933	1		130	12.8	0.5	1.15	0.090	0.-1.	8–9
No. 1 diluted with water to 2000 ml; cooled to 10°C. and poured into 2000 ml. of cold 2.5 N sulfuric acid with mechanical stirring; pH (electrometric by hydrogen electrode) = 0.02. A light precipitate formed	2	0	4000	0.416	0.016	0.0374	0.090		
5 ml. suspension No. 2 + 5 ml. 1.3 N sodium acetate	3	0.03	8000	0.19	0.02	0.0207	0.11		
5 ml. suspension No. 2 + 5 ml. 1.3 N sodium acetate	4	2				0.0228	0.12		
5 ml. suspension No. 2 + 5 ml. 1.3 N sodium acetate	5	19		0.19	0.02	0.0272	0.14		
Aliquot of suspension filtered	6								
5 ml. filtrate No. 6 + 5 ml. 1.3 N sodium acetate	7	19		0.044	0.036	0.010	0.23		
5 ml. suspension + 5 ml. 1.3 N sodium acetate	8	43		0.18	0.03	0.030	0.17		
5 ml. filtrate + 5 ml. 1.3 N sodium acetate	9	43		0.05	0.03	0.0108	0.22		
5 ml. suspension + 5 ml. 1.3 N sodium acetate	10	67		0.18	0.03	0.030	0.17		
5 ml. filtrate + 5 ml. 1.3 N sodium acetate	11	67		0.04	0.036	0.0115	0.29		
Suspension filtered after 67 hours in acid	12								
Filtrate No. 12 + 950 ml. saturated sodium acetate pH 2.5; + 2000 ml. saturated magnesium sulfate; filter. Residue dissolved in acetate buffer	13			9.5	1.9	1.9	0.20	0.-1.	3–4
$[\alpha]_{D\ pH\ 4.5}^{23°C} = 90$. Crystals were indistinguishable under the microscope from pepsin									
Residue No. 12 dissolved in water + sodium acetate pH 3.8	14		800						

TABLE VI—*Concluded*

Procedure	No.	Time in acid	Volume	N/ml.		$[\text{P.U.}]^{\text{Hb}}$		NH_2-N No./mol.	Acetyl No./mol.
				Protein	Non-protein	1/ml.	1/mg. P.N.		
		hrs.	*ml.*	*mg.*	*mg.*				
No. 14 cooled to 10°C. and added to 800 ml. cold 2.5 N sulfuric acid; a light precipitate formed	15		1600						
5 ml. suspension No. 15 + 5 ml. 1.3 N sodium acetate	16			0.270	0.018	0.048	0.18		
5 ml. suspension + 5 ml. 1.3 N sodium acetate	17	16		0.28	0.012	0.048	0.17		
Aliquot of suspension filtered	18								
5 ml. filtrate No. 18 + 5 ml. 1.3 N sodium acetate	19	115		0.038	0.018	0.0091	0.24		
5 ml. suspension + 5 ml. 1.3 N sodium acetate	20	115		0.110	0.020	0.0216	0.20		
5 ml. suspension + 5 ml. 1.3 N sodium acetate	21	210		0.099	0.03	0.021	0.21		
Suspension + 550 gm. sodium acetate; saturated with crystalline magnesium sulfate; filtered	22								
Residue No. 22 dissolved in acetate buffer; + equal volume of saturated magnesium sulfate; filtered. Residue = 15 gm., dissolved in water + acetate buffer, pH 4.5	23		25	17.7	0.8	3.5	0.20	2	2

carrying on the hydrolysis at 10°C. there was practically no loss of protein in 10 days. Table VI contains the description and results of an experiment on the acid hydrolysis of an acetylated pepsin preparation. The results show that with the removal of acetyl groups there was an increase in specific activity. The specific activity rose to that of pepsin where it remained practically constant. The acetyl content of the protein changed from eight or nine acetyl groups to three or four. Since there were still no free amino groups it is probable that these three or four acetyl groups are on the primary amino groups of pepsin and that this compound, therefore, corresponds to the "100 per cent active" acetyl pepsin prepared directly by slow acetylation of pepsin. On subjecting this material to a much longer treatment with acid a substance was obtained which has the same specific activity but now has two free amino groups and only two acetyl groups per molecule.

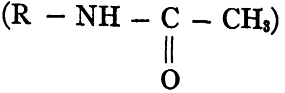
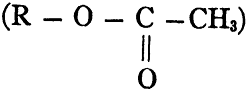
It is interesting to note that in general hydrolysis of acetyl groups linked to nitrogen atoms ( $R-NH-\underset{\underset{O}{\|}}{C}-CH_3$) requires very strong acid or alkali and high temperatures, whereas acetyl groups linked to oxygen ( $R-O-\underset{\underset{O}{\|}}{C}-CH_3$) are hydrolyzed with comparative ease.

TABLE VII

Specific Activity of "60 Per Cent Active" Acetylated Pepsin by Different Methods

Substrate	Method	Crystalline pepsin $[P.U.]_{mg.\ P.N.}$	Crystalline "60 per cent active" acetyl pepsin $[P.U.]_{mg.\ P.N.}$
Hemoglobin	Colorimetric estimation of liberated tyrosine	0.23	0.13
Casein	Viscosity (increase)	310	190
	Viscosity (decrease)	1700	850
	Solution	0.55	0.25
Edestin	Viscosity (decrease)	2200	1100
	Formol	7×10^{-3}	4×10^{-3}
Gelatin	Viscosity (decrease)	24	17
Rennet	Viscosity (increase)	33×10^{4}	23×10^{4}

General Properties

Activity by Various Methods.—The specific activity ($[P.U.]_{mg.\ P.N.}$) of a preparation of "60 per cent active" acetyl pepsin was determined by several different methods as described by Northrop (17). Although the specific activity of pepsin has been determined previously, using these same methods, the measurements are not all precisely reproducible (owing to variations in the substrate preparations) so that the activity of a standard pepsin solution was measured at the same time. The results given in Table VII show, within the limits of the methods, that the "60 per cent active" acetyl pepsin is a little over half as active as pepsin, by all of the methods tried.

pH Activity Curves.—It was thought that the shape of the pH activity curve of pepsin might be altered when the enzyme was acetylated. The pH activity curves of "60 per cent active" acetyl pepsin and of pepsin were determined by three different methods. The results in Fig. 8 show that the shape of the pH-activity curve of pepsin is not changed by acetylation of pepsin.

Experimental Procedure

1. Enzyme Concentration.—The enzyme solutions were diluted for each measurement so that the hydrolysis of the protein was carried to the same extent in each case. The activity was calculated as the reciprocal of the concentration of enzyme required to cause the same rate of hydrolysis at the various pH and is expressed as per cent of the maximum activity.

2. Substrates.—Aliquots of dissolved Hoffmann La Roche crystalline edestin and dialyzed Ox carbon monoxide hemoglobin solutions were added to equal volumes of hydrochloric acid of different concentrations. The pH of the solutions were determined electrometrically by a hydrogen electrode. The final concentration of edestin was 3 per cent and of hemoglobin was 2 per cent.

3. Methods—

(*a*) Hemoglobin:

1. Phenol-colorimetric, see Anson and Mirsky (18).

2. Non-protein nitrogen. 1 ml. of enzyme solution was added to 5 ml. of hemoglobin in a 50 ml. test tube. After 5 minutes the reaction was stopped by adding 10 ml. of M/5 pH 7.3 phosphate buffer from another 50 ml. test tube. The solution was mixed quickly by pouring back and forth from one tube to the other and then heated to boiling for 1 minute after which it was cooled quickly. This suspension was filtered through a Whatman No. 42 filter paper and refiltered if the filtrate was not perfectly clear. 5 ml. of the filtrate was analyzed by the Kjeldahl method for total nitrogen (19).

(*b*) Edestin:

1. Non-protein nitrogen. 20 ml. of 3 per cent edestin solution and 5 ml. of enzyme solution were brought to 35.5°C. in two 50 ml. test tubes and then mixed by pouring from one test tube to the other. After mixing, 5 ml. of the mixture was pipetted into each of three 50 ml. test tubes. The hydrolysis in the tubes was stopped after 1, 5, and 10 minutes by the addition and subsequent mixing of 5 ml. of 0.1 saturated sodium sulfate solution. After allowing these solutions to stand 20 minutes they were filtered through Whatman's No. 42 filter paper and 5 ml. of the filtrate analyzed for total nitrogen. The results of the three determinations at each pH were plotted, the slope determined and then corrected for the enzyme concentration. These corrected figures are plotted in Fig. 7 as per cent maximum activity against the pH.

Isoelectric point.—Since the primary amino groups are altered in the acetylation of pepsin one might expect a difference between the isoelectric points of the acetylated products and the original pepsin. Northrop (16) has found from minimal solubility and cataphoresis experiments that the isoelectric point of pepsin is close to pH 2.7. Microscopic examination (20) of an amorphous suspension of "60 per cent active" acetyl pepsin in hydrochloric acid of different concentrations revealed no change in the direction of migration. The particles of acetyl pepsin migrated to the anode at pH 3.0 and at pH 0.0 showing that within this range of pH the enzyme was negatively charged. The minimal solubility was determined for the "60 per cent active" and for the "100 per cent active" acetyl pepsins. In these experiments a large excess of solid material was present. The results plotted in Fig. 9 represent the solubilities of the amorphous materials in sulfuric acid solutions.

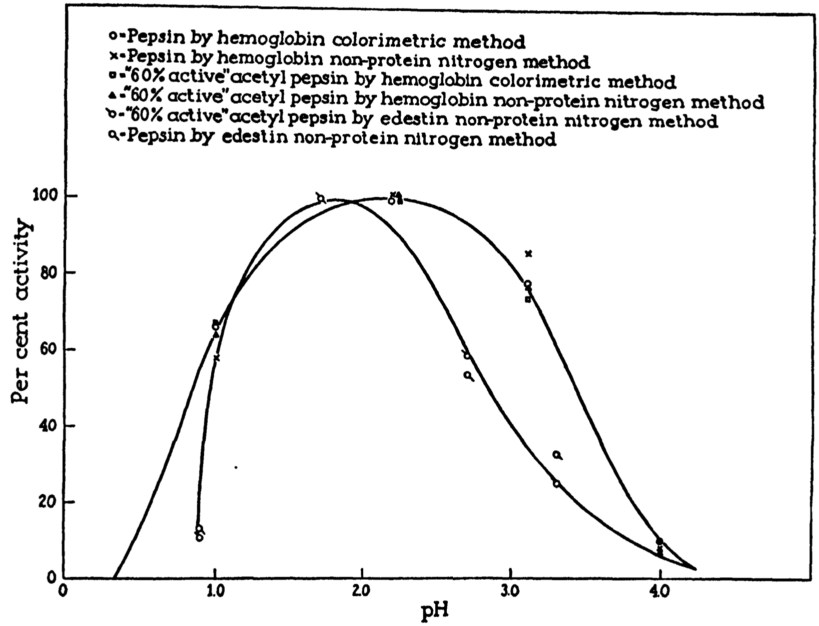


FIG. 8. Percentage activity at various pH of pepsin and "60 per cent active" acetyl pepsin.

Experimental Procedure

Enzyme Solutions.—

(*a*) "100 per cent active" acetyl pepsin Dec. 13, 1933, containing 8.7 mg. PN/ml. and less than 2 per cent non-protein nitrogen.

(*b*) "60 per cent active" acetyl pepsin Dec. 13, 1933, containing 16 mg. PN/ml. and 4 per cent non-protein nitrogen.

Procedure.—3 ml. of enzyme solution was added to 10 ml. of sulfuric acid of various concentrations at 23°C. with rapid stirring in a 50 ml. centrifuge cup. After stirring for 1 minute they were centrifuged and the clear supernatant liquid analyzed for nitrogen, activity, and pH. Stirring for a longer time did not alter the solubility appreciably. The pH was determined electrometrically with a hydrogen electrode.

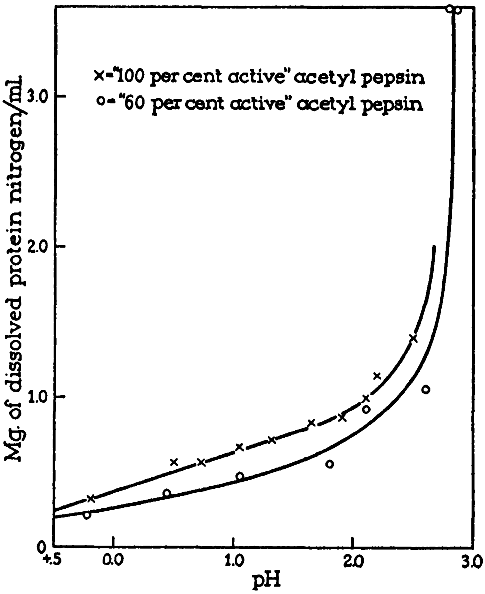


FIG. 9. Solubility of amorphous "100 per cent active" and "60 per cent active" acetyl pepsin in various concentrations of sulfuric acid.

The results in Fig. 9 show no minimal solubility but a very gradual decrease in solubility as the pH decreases. These results confirm the cataphoresis experiments in showing that the acetylated products have no isoelectric point.

Titration Curves.—The titration curves of pepsin and three of the acetylated products were determined, corrected, and are plotted in Fig. 10. In titrating the "10 per cent active" acetyl pepsin, constant E.M.F. readings could not be obtained in solutions more alkaline than pH 9.0. The drift was toward the acid region which seems to indicate that some hydrolysis takes place in solutions more alkaline than pH 9.0 and the hydrolysis products combine with the alkali. Up to pH 9.0 the E.M.F. readings were perfectly constant.

It might be expected that acetylation of the amino groups in pepsin would result in a difference in the titration curves at approximately pH 8.0 where the free amino groups usually titrate. This difference, however, of four amino groups per molecule is only 0.05 millimol of combined sodium hydroxide per gram of protein which could not be detected with any degree of certainty.

Experimental Procedure

The crystal filter cakes of the different acetyl preparations were washed a number of times with half saturated magnesium sulfate and while on the funnel from the last filtration were washed with small quantities of dilute hydrochloric acid until the filtrates gave no sulfate precipitate on the addition of barium ion. The hydrochloric acid was washed out with N/10 potassium chloride solution. 10–15 gm. of filter cake was then stirred to a paste and diluted to 50 ml. with N/10 potassium chloride solution and the suspension placed in a cell connected to a calomel half cell by means of a saturated potassium chloride-agar bridge. The solution was stirred rapidly but did not foam. 2.05 N sodium hydroxide was run into the suspension from a 5 ml. buret through a long capillary extending under the surface of the solution. A hydrogen electrode, standardized before and afterwards with N/10 hydrochloric acid was used in the determination of E.M.F. When the solution reached pH 12.0 the volume and nitrogen content were determined.

Enzyme Preparations Used for Titration Curves. 1. Pepsin.—Twice crystallized pepsin No. 1 of Nov. 21, 1933, contained 10 per cent non-protein nitrogen. 4.8 gm. of pure protein calculated from the nitrogen analysis was used.

2. "100 Per Cent Active" Acetyl Pepsin.—No. 3 of Dec. 7, 1933, contained 4 per cent non-protein nitrogen; 2.8 gm. of pure protein was used.

3. "60 Per Cent Active" Acetyl Pepsin.—No. 13 of June 12, 1933, and recrystallized on Dec. 10, 1933, contained 3 per cent non-protein nitrogen. 3.5 gm. of pure protein was used.

4. "10 Per Cent Active" Acetyl Pepsin.—Dec. 28, 1933, contained 4 per cent non-protein nitrogen; 4.3 gm. of pure protein was used.

Acid and Alkali Inactivation.—In studying the acid and alkali inactivation of acetylated pepsins it was hoped that some difference would develop which would indicate a new characteristic property produced by the introduction of acetyl groups into the pepsin molecule. The results are graphically represented in Figs. 11 and 12. In Fig. 11 the points for pepsin were taken from Northrop (21). The procedure in the experiment on the acid inactivation of acetylated pepsin was the same as that used with acid inactivation of pepsin (21) so that the results may be compared. The change in activity could not be used as a measure of the loss in protein in the acid inactivation of acetylated pepsin for in strong acid the acetyl groups are hydrolyzed resulting in an increase in the specific activity of the protein.

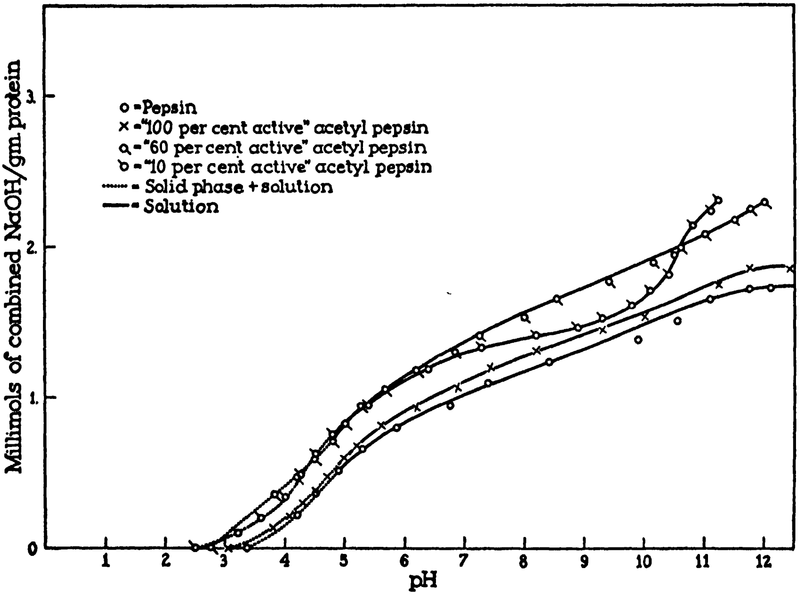


FIG. 10. Titration curves of pepsin and three different acetyl derivatives of pepsin at 20°C.

Experimental Procedure

1. Acid Inactivation.—5 gm. of once crystallized "60 per cent active" acetyl pepsin filter cake was dissolved in 250 ml. of water with the aid of sodium acetate. 5 ml. of this solution was added to 20 ml. of various concentrations of hydrochloric acid; the solutions were kept at 35°C. and analyzed immediately and after 24 hours for protein nitrogen.

2. *Alkali Inactivation.*—The enzyme solutions were made up to contain 3 mg. PN/ml. 1 ml. of this enzyme solution was then added to 5 ml. of M/1 phosphate buffer at various pH and allowed to stand at 23°C. After 5 minutes 5 ml. of this solution was titrated with 5 ml. of sulfuric acid of a concentration to bring the resulting solution to pH 6.0. 1 ml. was diluted for activity measurements by the

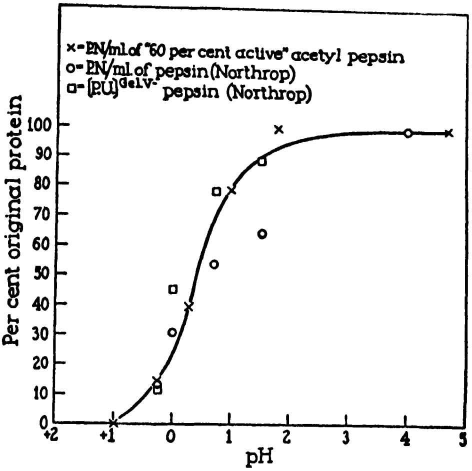


FIG. 11. Per cent inactivation of pepsin and "60 per cent active" acetyl pepsin in various concentrations of hydrochloric acid at 35°C.

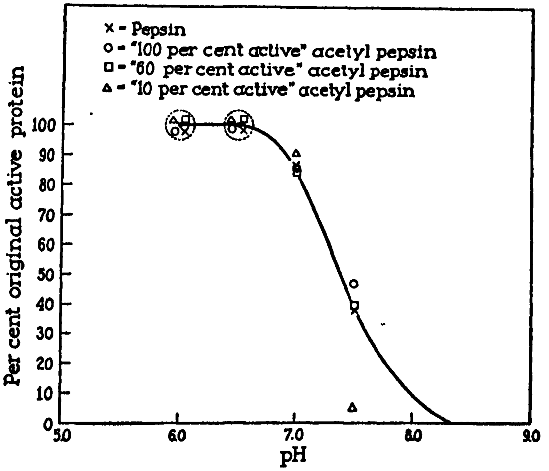


FIG. 12. Per cent inactivation of pepsin and three different acetyl derivatives of pepsin in phosphate buffer at various pH and at 23°C.

hemoglobin method, the dilutions being so arranged that approximately the same colorimeter reading was obtained.

The results plotted in Figs. 11 and 12 show that the acid and alkali inactivation of acetylated pepsin solutions follow approximately the same course as that of the parent protein, pepsin.

One point might be mentioned which is not revealed in the figures and which, though not a difference in the proteins is of some interest. A few moments after the acetylated pepsin was mixed with the 6 M hydrochloric acid a precipitate formed which remained during the 24 hours of the experiment. This precipitate was approximately 80 per cent of the original protein. It was completely inactive and insoluble under conditions in which the original material was active and soluble. It was found to be denatured protein. Northrop (21) anticipated the presence of denatured pepsin protein in strong acid solutions but failed to find it. It appears that his solutions were not carried to concentrated enough acid for on similarly treating pepsin with 6 M hydrochloric acid the protein precipitated as denatured protein.

EXPERIMENTAL METHODS

Acetylation with Ketene

The ketene used throughout this work was prepared by the thermal decomposition of acetone by means of a generator described by Herriott (14). A 500 ml. round bottom flask was employed to hold the acetone. A few alundum chips prevented superheating and bumping of the acetone as it was heated on a steam bath. A long water condenser followed by an ice trap removed most of the polymers of ketene and the escaped acetone vapor. The ketene was passed into the pepsin solution through an inverted 25 mm. funnel. It was found that a 500 ml. wide mouth Erlenmeyer flask was the most convenient container for the enzyme solution since it allowed the ketene to be introduced, the solution to be stirred with a mechanical stirrer, and samples to be removed without interference. During long (10 hour) exposures to ketene the Erlenmeyer flask was kept in water at 25°C. ±2° for there is some heat developed on the introduction of ketene into the solution.

pH Determinations

In general the pH estimations were made by the colorimeter method using the indicators recommended by Clark (22).

Activity Determinations

Unless otherwise specified the activity determinations throughout this paper were made by the colorimetric method of Anson and Mirsky (18).

Nitrogen Determinations

Protein nitrogen has been determined as the difference between the total nitrogen and the non-protein nitrogen. This is justifiable when the non-protein nitrogen is a small part of the total nitrogen. The enzyme solutions were diluted to contain 1–2 mg. of nitrogen per ml.; 1 ml. was used for the total nitrogen; 2 ml. was placed in a 50 ml. centrifuge cup and 10 ml. of boiling 5 per cent trichloroacetic acid added; the cup was shaken a moment and placed in a beaker of cold water. After cooling the suspension was filtered and 6 ml. of filtrate used for nitrogen analysis. This is the non-protein nitrogen. All nitrogen determinations were made by the micro Kjeldahl method as described by Northrop and Kunitz (19).

Acetyl Estimation

A volume of enzyme solution containing 0.5–0.75 gm. of protein was placed in a 150 ml. beaker and this in turn placed in water at approximately 45°C. At least five times that volume of boiling 5 per cent trichloracetic acid was added and the beaker placed immediately in ice water and the solution stirred rapidly. This treatment precipitates the protein and cools the resulting suspension in a very short time. After cooling the suspension was filtered without suction and the precipitate washed with distilled water until the filtrate was blue when tested with the indicator brom cresol green indicating that the filtrate was pH 5.0 or more. This usually required from 15–20 washings with water. The washing removed the acetic acid which had been present in the solution as acetate buffer but was converted to acetic acid by the excess trichloracetic acid. The denatured protein is completely insoluble under these conditions. When the precipitate had been washed free of acid most of the water was drawn off with the aid of suction and the precipitate dissolved in a small amount of dilute alkali or sodium phosphate. After the precipitate had dissolved completely 1 ml. of 4 M sodium hydroxide was added and 1 ml. of solution withdrawn and analyzed for nitrogen from which the protein content was calculated. The remaining liquid (approximately 11 ml.) was put into a test tube and allowed to stand at 35°C. for at least 5 days. After this time 10 ml. was put into a 150 ml. modified Claissen distilling flask with 5 ml. of 85 per cent phosphoric acid (reagent grade) and 0.2 ml. of octyl alcohol. The alcohol prevents foaming of the protein which would otherwise render vacuum distillation impossible. The distilling flask was kept at 50–60°C. on a water bath while the receiving flask was cooled with running water. Distillation was carried out for 15 minutes at 20 mm. of mercury pressure. At the end of this time the receiving flask was removed, the sides washed down with a few ml. of water, a drop of 0.5 per cent phenolphthalein added, and the solution titrated to a faint pink with N/50 sodium hydroxide. As a check on the titration figure and on the identity of the acid as acetic acid a drop of 0.1 per cent brom cresol green was added and the solution titrated back to pH 4.7 corresponding to the p*K* of acetic acid, with N/50 hydrochloric acid. The second titration will be half of the first titration if only acetic acid is present in the distillate. After the titration the receiving flask was rinsed out with distilled water and fitted onto the distilling flask. 5 ml. of water and 0.2 ml. of octyl alcohol were added to the distilling flask and the distillation, followed by the titration, was carried out as before. This was repeated a third time which was usually sufficient to carry over nearly all of the acetic acid. A blank of 0.10 ml. of N/50 sodium hydroxide for each distillation and titration was deducted from the total titration value before the amount of acetic acid distilled over was calculated.

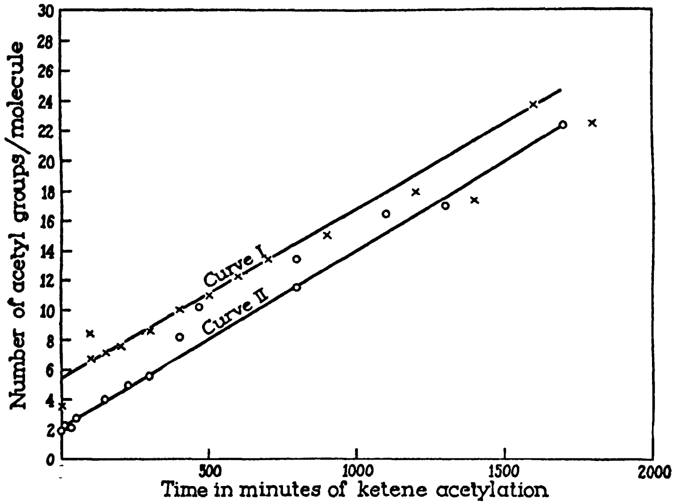


FIG. 13. Change in acetyl groups per molecule of protein during acetylation.

The above procedure was used for most of the acetyl estimations but in the case of pepsin a small amount of trichloracetic acid survived the washing and distilled over. This acid was probably combined with the free NH_2 groups as a salt. It was detected by the second or back titration to pH 4.7 When dilute sulfuric acid was used in place of trichloracetic acid this difficulty was avoided.

To illustrate the precision of this method of acetyl estimation the increase in acetyl groups per molecule is plotted against the time of acetylation in Fig. 13. Curves I and II were obtained from the same experiments as Curves I and II of Fig. 3 were.

Acetyl estimations were made on pepsin and acetylated pepsin by two methods other than the one described above. One of the two methods consisted in heating a mixture of 5 ml. of protein solution and 50 ml. of a 25 per cent solution of *p*-toluenesulfonic acid to 110°C. for 6 hours under a reflux condenser followed by the usual vacuum distillation and titration of the liberated acetic acid. In the other procedure the protein, in 0.4 N NaOH, was heated to 100°C. under a reflux condenser for varying periods of time up to 5 days and the liberated acetic acid determined as in the other methods after acidifying the alkaline digest. 2 ml. of molar cadmium sulfate was introduced into the distilling flask to retain any hydrogen sulfide, which is liberated on acidification of the digest. The results of the three methods agreed although by the last method a slightly higher acetyl figure was obtained for both pepsin and the acetylated pepsin but the difference agreed with the figures obtained by the other two methods.

Amino Nitrogen

Pepsin decomposes rapidly into non-protein split products which have a high amino nitrogen content and which can be removed only with difficulty. The pepsin protein was therefore denatured, washed, and dissolved before analyzing for amino nitrogen. It was denatured by adding five times its volume of boiling 5 per cent trichloracetic acid and the resulting suspension cooled quickly in cold water. After washing the precipitate at least three times with several volumes of water or 2.5 per cent trichloracetic acid the residue was dissolved in dilute alkali or phosphate buffer. The pH of the dissolved denatured protein was below pH 8.0. These solutions contained from 40–60 mg. of protein per ml. An aliquot of the solution was analyzed for total nitrogen from which the protein content was calculated. The amino nitrogen was determined on the Van Slyke micro apparatus (15). The nitrous acid-protein mixtures were shaken for 20 minutes. Most of the amino nitrogen estimations were made on solutions of the washed trichloracetic acid precipitate. These analyses were easily reproducible and agreed with the figures obtained by analysis of native pepsin which had been washed with half saturated magnesium sulfate and which contained less than 3 per cent non-protein nitrogen. The figures obtained from analyses of these trichloracetic acid precipitates were never higher than those obtained with native pepsin. This result shows that no hydrolysis of the protein occurs during denaturation unless there is a simultaneous coupling of an amino group at some other point. The amino nitrogen content for native and denatured pepsin was also determined by Sörensen's formol titration as modified by Northrop (23) and found to agree with the values obtained by the Van Slyke nitrous acid method.

SUMMARY

Crystalline pepsin has been acetylated by the action of ketene in aqueous solution at pH 4.0–5.5. As acetylation proceeds the activity decreases, the decrease being more rapid at pH 5.0–5.5 than at 4.0–4.5.

Three acetyl derivatives have been isolated from the reaction

mixture and obtained in crystalline form. The crystal form of these derivatives is similar to that of pepsin.

Fractionation and solubility determinations show that these preparations are not mixtures or solid solutions of the original pepsin with an inactive derivative.

A compound which contains three or four acetyl groups and which has lost all of its original primary amino groups can be isolated after short acetylation. It has the same activity as the original pepsin. A second derivative containing six to eleven acetyl groups has also been isolated. It has about 60 per cent of the activity of the original pepsin. A third derivative having twenty to thirty acetyl groups and about 10 per cent of the activity of original pepsin can be isolated after prolonged acetylation. The 60 per cent active derivative on standing in strong acid solution loses some of its acetyl groups and at the same time regains the activity of the original pepsin. The compound obtained in this way is probably the same as the completely active three acetyl derivative obtained by mild acetylation. These results show that acetylation of three or four of the primary amino groups of pepsin causes no change in the specific activity of the enzyme but that the introduction of acetyl groups in other parts of the molecule results in a marked loss in activity.

The solubilities, amino nitrogen content, acetyl content, isoelectric point, and the specific activity have been determined by a variety of methods and found to be different from the corresponding properties of crystalline pepsin. The pH-activity curves, acid and alkali inactivation, and titration curves were not significantly different from the same respective properties of pepsin.

REFERENCES

1. Sumner, J. B., *J. Biol. Chem.*, 1926, **69**, 435.
2. Northrop, J. H., *J. Gen. Physiol.*, 1930, **13**, 739.
3. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1932, **16**, 267.
4. Kunitz, M., and Northrop, J. H., *Science*, 1933, **78**, 558.
5. Caldwell, M. L., Booher, L. E., and Sherman, H. C., *Science*, 1931, **74**, 57.
6. Warburg, O., and Christian, W., *Biochem. Z.*, Berlin, 1933, **266**, 377.
7. Hunt, R., *J. Pharmacol. and Exp. Therap.*, 1915, **7**, 305.
8. Warburg, O., and Negelein, E., *Biochem. Z.*, Berlin, 1932, **244**, 20.
9. Freudenberg, K., and Dirscherl, W., *Z. physiol. Chem.*, 1928, **175**, 1; 1930, **187**, 89. Freudenberg, K., and Eyer, H., *Z. physiol. Chem.*, 1932, **213**, 226.

10. Avery, O. T., and Goebel, W. F., *J. Exp. Med.*, 1933, **58**, 731.
11. Hugounenq, L., and Loiseleur, J., *Bull. soc. chim. biol.*, 1925, **7**, 955.
12. Staudinger, H., Die Ketene, Stuttgart, F. Enke, 1912.
13. Bergmann, M., and Stern, F., *Ber. chem. Ges.*, 1930, **63**, 437.
14. Herriott, R. M., *J. Gen. Physiol.*, 1934, **18**, 69.
15. Van Slyke, D. D., *J. Biol. Chem.*, 1913, **16**, 121.
16. Northrop, J. H., *J. Gen. Physiol.*, 1930, **13**, 767.
17. Northrop, J. H., *J. Gen. Physiol.*, 1932, **16**, 41.
18. Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1932, **16**, 59.
19. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1932, **16**, 313.
20. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925, **7**, 729.
21. Northrop, J. H., *J. Gen. Physiol.*, 1932, **16**, 33.
22. Clark, W. M., Determination of hydrogen ions, Baltimore, The Williams & Wilkins Co., 3rd edition, 1928.
23. Northrop, J. H., *J. Gen. Physiol.*, 1926, **9**, 767.
24. Tauber, H., and Kleiner, I. S., *J. Biol. Chem.*, 1934, **104**, 261.

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A CONVENIENT KETENE GENERATOR

BY ROGER M. HERRIOTT

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Accepted for publication, April 21, 1933)

A simple apparatus for the preparation of ketene ($CH_2=C=O$) from acetone vapor is described by Ott, Schröter, and Packendorff (1). A more rigid and convenient apparatus has been designed and used by the writer (Fig. 1). Pyrex glass used in both the filament-support and bulb reduces the possibility of cracking or breaking. The removable filament-support makes it possible to replace the filament in a few moments. By turning up the ends of the 30 mill tungsten leads sealed into the glass, one may wind on the platinum or tungsten wire and thus rapidly replace the filament.

Platinum wire (Brown and Sharpe gauge No. 30) across a 110 volt circuit gives satisfactory results. A resistance in series with the lamp regulated to let 2 to 6 amperes through the filament results in a rapid evolution of ketene: After 8 to 12 hours continuous use there is a carbonaceous deposit on the filament. This deposit, being extremely brittle, may be removed by drawing the platinum filament over any sharp edge. The wire is then ready to be used again. A duplicate support and filament may be constructed which will be ready to replace the one in use if it burns out or breaks.

REFERENCE

1. Ott, E., Schröter, R., and Packendorff, K., *J. prakt. Chem.*, 1931, **130**, N.S., **177**.

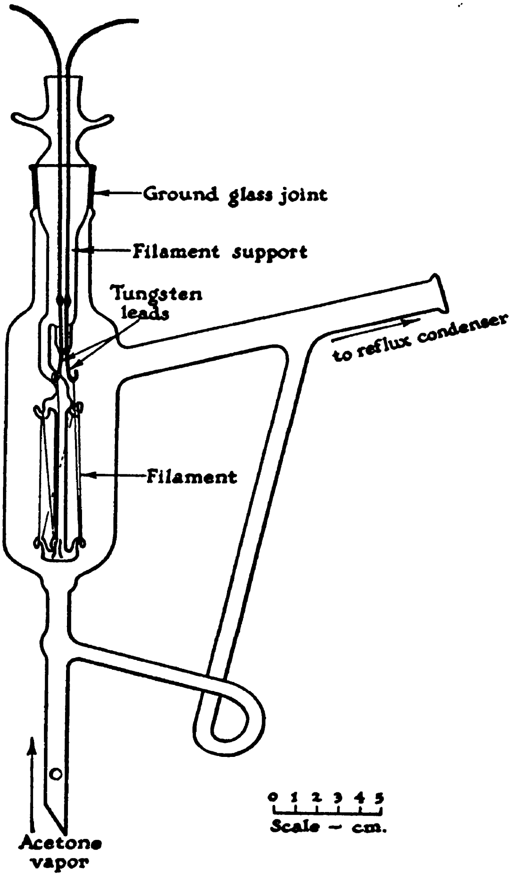
Ground glass joint
Filament support
Tungsten leads
to reflux condenser
Filament
Acetone vapor
0 1 2 3 4 5
Scale — cm.

FIG. 1

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, September, 1934, Vol. 106, No. 2, pp. 605–614]

A STUDY ON KERATIN

BY DAVID R. GODDARD* AND LEONOR MICHAELIS

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, May 16, 1934)

Keratins are the proteins of epidermal and skeletal tissues which are insoluble in the usual protein solvents, not digested by trypsin or pepsin, and high in cystine content. Such a definition intentionally excludes fibroin, the major protein of silk. It will be shown in this paper that keratins can be converted into proteins soluble in alkali or acid, with a definite optimum pH of flocculation (which may be interpreted as an isoelectric point), and digestible by trypsin or pepsin. This is accomplished by breaking the disulfide bonds of the protein. Papers on the oxidation of keratins have been published by Lissizin (1), Stary (2), and Waldschmidt-Leitz (3), who used bromine, permanganate, and H_2O_2 as oxidants. Stary and Waldschmidt-Leitz have shown that the oxidized keratin is digested by trypsin. The oxidizing agents are not specific for the disulfide groups, but attack the protein molecule at other points, and they act very slowly. In contrast, the reductants will be shown to act very quickly and without bringing about any other appreciable chemical alteration than that concerned with the sulfur. These agents dissolve keratin only at alkaline reaction (pH 10 to 13), but the action is not due to alkali alone. Products prepared from the solutions behave as true proteins, and not as products of hydrolysis. Their solutions are precipitated by ordinary protein precipitants such as sulfosalicylic acid and lose this property when digested by trypsin or pepsin.

Reductants available for reduction of disulfide groups are thioglycolic acid, potassium cyanide, sodium sulfide, and sodium sulfite. The chemical process exhibited by these reagents on simple disulfide compounds such as cystine can be formulated as follows:

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* National Research Council Fellow in the Biological Sciences.

(1) $R{-}S{-}S{-}R + 2HS{-}CH_2COOH \rightarrow 2R{-}SH + [S{-}CH_2COOH]_2$ (see (4))*
(2) $R{-}S{-}S{-}R + HCN \rightarrow R{-}SH + R{-}S{-}CN$ (see (5))
(3) $R{-}S{-}S{-}R + 2H_2S \rightarrow 2R{-}SH + H_2S_2$†
(4) $R{-}S{-}S{-}R + H_2SO_3 \rightarrow R{-}SH + R{-}S{-}S{-}O_3H$ (see (6, 7))

Reactions 1 to 3 occur only in slightly alkaline solution; Reaction 4 is most rapid in neutral to mildly acid solutions.

When keratin, such as wool or feathers, is treated with thioglycolic acid at a pH of 10 or higher the reaction appears to be identical with that on simpler disulfides. The reaction is a simple reduction; no loss of sulfur occurs. The sulfhydryl protein can be reoxidized to the disulfide state, and this disulfide protein is still soluble in acid or alkali and digestible by trypsin or pepsin.

The action of cyanide on wool is not quite so simple. A higher alkalinity is required (pH 12 to 13) and the proteins lose sulfur; however, the substances obtained behave as true proteins, not as products of hydrolysis.

The dissolving action of sodium sulfide has been known for a long time and is used industrially. Küster and coworkers (8) and Merrill (9) have considered the dissolving of keratin as a hydrolysis. Pulewka (10) and Speakman (11) realized that the action is on the disulfide groups. However, substances obtained behave as true proteins. In contrast to the cyanide preparations they contain more sulfur than the native keratin, though the cystine determinations by the Folin and Marenzi (12) method give the same values as the wool. It is likely that the action of Na_2S on keratins is not identical with its action on simpler disulfides and seems to be analogous to the action of sulfite on cystine, and a polysulfide is formed which behaves as cysteine in the Folin and Marenzi procedure. When such a protein is redissolved in weak alkali and reprecipitated with acid, it undergoes a loss in sulfur, but always in such a way that the total sulfur exceeds the cystine sulfur calculated from the cystine determination on the same preparation.

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* Dr. A. E. Mirsky called our attention to the use of thioglycolic acid as a protein reductant. Unpublished work from this laboratory has shown that cystine is reduced at mildly alkaline reaction by an excess of thioglycolic acid. The cysteine has been almost quantitatively recovered by converting it to benzylcysteine.

† Unpublished work from this laboratory has shown that cystine is reduced according to Reaction 3. The cysteine and disodium-disulfide have been recovered as benzyl derivatives.

Sodium sulfite does not dissolve wool at an appreciable rate at any pH; so Reaction 4 has no practical significance for keratin.

EXPERIMENTAL

Most of the experiments were performed with native sheep wool, freed from fat by ether extraction. Other experiments with chicken feathers gave similar results. The wool was analyzed for cystine by the Folin and Marenzi (12) procedure, for nitrogen by the Kjeldahl method, and for sulfur by bomb combustion (13). The results, corrected for moisture, but not for ash, are as follows: N 16.66, S 3.19, cystine 11.90, 11.86 (calculated according to sulfur content, 11.9).

Protein Obtained with Thioglycolic Acid

50 gm. of wool were dissolved in 2 liters of a 0.5 M thioglycolate solution. This solution was prepared by neutralizing 92 gm. of thioglycolic acid with twice the amount of concentrated NaOH necessary to neutralize it to the turning point of phenol red, and made up to 2 liters. (The pH of the solution is about 12.) After 3 hours at 30° practically all the wool had dissolved. The mixture was filtered and the filtrate precipitated with 2 moles of glacial acetic acid, the precipitate collected on a filter, immediately twice ground with acetone made acid with HCl, washed with ether, and dried *in vacuo*.

The yield was 30 gm. of dry white powder which was still contaminated with a small amount of thioglycolic acid. The protein was freed of the last traces of thioglycolic acid by suspending in water and dialyzing for 3 days against distilled water in a cellophane tube. The fact that the protein was free from thioglycolic acid was shown by the cobalt test described in the appendix. The protein obtained is insoluble in water or neutral salts, but is soluble in strongly acid or alkaline solutions. The dialyzed proteins gave directly a strong nitroprusside test. They are slowly oxidized by air in bicarbonate solutions. Then the nitroprusside test of the protein is positive only after addition of KCN. The oxidized protein can be reduced over again by thioglycolic acid in bicarbonate buffers.

Four preparations have been made from wool by treatment for 1, 3, 6, and 24 hours with thioglycolate solutions. No increased yield is obtained after 3 hours. On varying the pH of the thioglycolate the wool does not dissolve at pH 9.0; it dissolves slowly at 10.1, and rapidly at a pH of 11 or over. Control experiments with M sodium carbonate or ammonium hydroxide showed that no dissolving action occurred in the absence of thioglycolate.

Analyses of two of the preparations are given below. The cystine determinations were made after first oxidizing the hydrolyzed protein with 3 per cent H_2O_2 (as Dr. A. E. Mirsky suggested to us) and then following the Folin-Marenzi method. The nitrogen determination was by micro-Dumas. The figures are in percentage of the dry weight and are not corrected for ash.

	Treated with thioglycolate for 24 hrs.	Treated with thiogycolate for 3 hrs.
Sulfur	3.20	3.13
Nitrogen	15.71	15.75
Cystine	11.8	11.9
" calculated from sulfur	12.0	11.74
Isoelectric point	4.6	4.7

Proteins Prepared by Potassium Cyanide

Wool does not appreciably dissolve in 1 M KCN solutions, even in days, and in such a solution it turns dark brown to black. In 0.1 N NaOH and M or 0.5 M KCN most of the wool dissolves in a few hours, and such solutions do not become black even after days. If the wool is first treated with NaOH, washed, and exposed to KCN, it does not dissolve. The preparation of the protein from the solution was analogous to that described above.

A protein was prepared from wool by treating 50 gm. of wool with 2 liters of M KCN in 0.1 N NaOH. The yield was 17.6 gm. of dry protein. The protein is similar to that obtained by thioglycolic acid. It is slightly more soluble, lower in sulfur and cystine content. It gives the nitroprusside test and does not lose this property even when its alkaline solution is exposed to the air for days. Preparations made by treatment with KCN for 1, 6, 24, and 48 hours had sulfur contents of 1.3, 1.7, 1.8, and 1.4 respectively.

Analyses of two preparations are given below.

	Treated with KCN for 24 hrs.	Treated with KCN for 6 hrs.
Sulfur	1.8	1.73
Nitrogen	15.99	14.79
Cystine		1.86, 1.87
Isoelectric point	4.7	4.7

Preparation with Sodium Sulfide

50 gm. of wool were dissolved in 2 liters of 0.5 M Na_2S. The protein was isolated as above. The S content was very high, due in part to free sulfur extractable by CS_2. However, the final preparations gave high constant values of S, and this could not be decreased by further grinding with CS_2. When the entire preparation of the protein is carried out under nitrogen, no free sulfur is formed, but the sulfur content of the protein is still high. The protein so isolated gave a negative nitroprusside test (even when prepared under nitrogen) unless first treated with KCN. The isoelectric zone is wide, but one preparation, as colloidal solution made by dialyzing the Na_2S-wool solution without previous precipitation, gave a sharp isoelectric point at pH 4.9.

The analyses of two preparations are given below.

	Sample A. Treated with Na_2S for 48 hrs.	Sample B. Treated with Na_2S for 6 hrs.
Sulfur	6.61, 6.55	5.36
Nitrogen	12.99	13.62
Cystine	11.63	11.4
Isoelectric point	4.1–4.5	4.2–4.6

Sample A was redissolved in concentrated NH_4OH, filtered, and reprecipitated with acetic acid. On analysis the results obtained were: S, 3.93; cystine, 6.94 (cystine S, 1.85). Both the original and the reprecipitated protein, though differing in total S, contain approximately twice the amount of sulfur as corresponds to the cystine sulfur.

Solubility and Flocculation

All the proteins described above are insoluble in water and resemble casein or denatured proteins. The dry proteins can best be dissolved in weak alkalies by grinding in a mortar with Na_2CO_3 or $NaHCO_3$. In dilute NaOH they dissolve readily, but lose sulfur. When the filtrate of wool dissolved in Na_2S is dialyzed directly for several days, the protein remains in solution, but it is precipitated by a trace of acetic acid. Among the dry proteins, the cyanide preparation dissolves most readily.

The isoelectric points were estimated by the method of Michaelis and Rona (14). The dry proteins were ground with Na_2CO_3 and water, and filtered, so that the solution was 0.1 M in Na_2CO_3 and 0.5 per cent in protein. To a series of test-tubes 1 cc. of protein solution and increasing amounts of acetic acid were added, so calculated that all the tubes had a total volume of 10 cc. and an arithmetical series of pH differing by 0.3 pH. The optimum of flocculation was taken as the isoelectric point, and its pH and that of the adjoining tubes was determined with the glass electrode. The results obtained are tabulated among the analyses above. The isoelectric points for these protein preparations are slightly lower than the isoelectric point of native wool. Speakman (15) and Elöd and Silva (16) by independent methods obtained the values of 4.8 and 4.9.

Digestion by Trypsin and Pepsin

Trypsin, purified by the method of Anson and Mirsky (17), was added to suspensions of the proteins in bicarbonate buffers at pH of 8.5 to 8.8, and maintained at 37°. Samples were withdrawn and precipitated with sulfosalicylic acid. The controls showed complete precipitation, and practically all of the nitrogen was in the precipitate. The digestion series showed progressively less precipitation, and after 2 hours 90 to 95 per cent of the nitrogen was in the filtrate. All of the proteins described above are digested by trypsin, and the thioglycolic acid preparation is digested when it is either in the sulfhydryl or the disulfide state.

The dry proteins were suspended in dilute HCl, pepsin was added, and the mixture had a pH of 1.30. The suspensions, maintained at 37°, gradually became clear due to digestion by the pepsin. These solutions remained clear upon the addition of sulfosalicylic acid. Nitrogen determinations on the filtrates of control showed practically complete precipitation, and on the digestion series about 80 per cent digestion. All the proteins described above were similarly digested, and the state of the proteins as sulfhydryl or disulfide did not affect the result.

DISCUSSION

The keratins and fibroin are similar in their insolubility in the usual protein solvents, and in their indigestibility by pepsin and trypsin, but that they are essentially different in structure is shown by the agents which bring about their solution. Fibroin, though not soluble in water, dilute acids, or alkali, is easily dissolved in concentrated solutions of certain neutral salts such as calcium sulfocyanate or lithium iodide (18) and also in cold concentrated hydrochloric acid. These solutions can be easily reprecipitated, and the precipitate shows a fibrous structure. Wool cannot be dispersed[1] in any of those solvents that act on silk, but it can be readily dissolved in a series of agents that split the disulfide bond, as alkaline solutions of sodium cyanide, sulfide, and thioglycolate. These solutions do not dissolve silk in a perceptible degree. The protein, reprecipitated from a solution of wool in one of these agents, is never fibrous but amorphous.

These properties are in good agreement with the known differences in chemical composition and structure of these two classes of proteins. The x-ray diffraction studies of Brill (19) and Meyer and Mark (20) show that fibroin consists of fully extended polypeptide chains, and these chains must be oriented parallel to each other chiefly by residual forces for fibroin contains no cystine and but very little diamino and dicarboxylic acids. The regularity of the fully extended chain of amino acids in silk seems to be sufficient to bring about a tight fiber structure, without lateral chemical links, but this is not the case in the keratins. The x-ray studies of Astbury (21) on mammalian hairs have shown that these proteins are crystalline with partially contracted polypeptide chains. Speakman (22) has pointed out the importance of the lateral links between parallel polypeptide chains, the bond of the disulfide group of cystine, and the polar link of diamino and dicarboxylic acids. The stability of the keratin molecule depends on these bonds. Once these two types of bridges have been broken by chemical attack, the keratins behave in their solubility and digestibility by proteolytic enzymes as denatured proteins.

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[1] Von Weimarn claims that wool can be dispersed in a concentrated boiling solution of calcium or lithium sulfocyanate at 180°. It is true that the wool dissolves very slowly, but the solution becomes alkaline and the presence of sulfides can be demonstrated. So the action can be best interpreted as a dissolution by alkaline sulfides formed from the slow decomposition of sulfocyanate.

The sulfhydryl protein, produced by the action of thioglycolic acid on wool, can be reoxidized to the disulfide state by air, in moderately alkaline solution, or by hydrogen peroxide. The reoxidized protein, in spite of its disulfide bonds, is still soluble in alkali, is enzyme-digestible, and has about the same isoelectric point. In the reoxidation of the sulfhydryl protein, the original crystalline pattern is not reformed, the disulfide bonds are no longer rungs of a ladder connecting parallel polypeptide chains. This shows that though the disulfide bond is essential for the properties of native keratin, these properties cannot be ascribed to the purely chemical character of this group. It is the physical pattern of the keratin, with a definite spatial arrangement of peptide chains and disulfide bonds, which imparts to keratin its resistance to enzyme hydrolysis and dissolving agents.

Mention should be made of the fact that all of the agents used to dissolve wool require a higher alkalinity than to reduce a simple disulfide such as cystine. The pH used is never high enough to dissolve wool of its own accord in a comparable time, yet the necessity of a distinctly alkaline reaction is striking and demands an explanation.

The following hypothesis may be offered. The cross links between peptide chains in keratin are of two kinds, disulfide links and bridges formed by the electrostatic attraction of the NH_3^+ group of the diamino acids for the COO^- group of the dicarboxylic acids. These salt-like bridges will be broken in alkaline solution by removal of a proton from the amino group. It appears that it is necessary to open these links before the disulfide groups may be reduced.

SUMMARY

Keratin dissolves in Na_2S, KCN, or thioglycolic acid at alkaline reaction. This effect is chiefly due to the splitting of the disulfide groups, which are essential for the maintenance of the fibrous structure of keratin. Chemically, the action of thioglycolic acid is the simplest; it simply reduces the disulfide to sulfhydryl groups with no other appreciable chemical change. The other reagents act in a more complicated way. The substances thus obtained are proteins. They are soluble in alkali or acid, with a definite isoelectric point, and they are digestible by pepsin and trypsin, even when secondarily the —SH group has been reoxidized to the —SS— stage, or when due to secondary reactions the sulfur content has been greatly changed.

Appendix

Test for Thioglycolic Acid—In order to test whether the protein prepared with thioglycolic acid is completely freed from this acid the following test was applied. A suspension of the protein in sodium pyrophosphate solution is mixed with a drop of a 1 per cent solution of cobalt sulfate. Any trace of free thioglycolic acid will develop, either immediately or after some time, on exposing the mixture to the air, a brown color. The sulfhydryl protein does not give this test, although it gives a positive nitroprusside test which is common for all sulfhydryl compounds. This test is based on the formation of strongly colored cobalti complexes of thioglycolic acid according to Michaelis and Schubert (23). The smallest amount of thioglycolic acid giving a distinct positive test in a volume of 1 cc. is approximately 0.05 mg. The oxidized form of thioglycolic acid, dithiodiglycolic acid, can be tested for with the same reagent, adding besides some Na_2SO_3 which reduces the disulfide to the sulfhydryl compound.

BIBLIOGRAPHY

1. Lissizin, T., *Z. physiol. Chem.*, **173**, 309 (1928).
2. Stary, Z., *Z. physiol. Chem.*, **136**, 160 (1924); **144**, 147 (1925); **175**, 178 (1928).
3. Waldschmidt-Leitz, E., and Schuckmann, G., *Ber. chem. Ges.*, **62**, 1891 (1929).
4. Hopkins, F. G., *Biochem. J.*, **19**, 787 (1925).
5. Mauthner, J., *Z. physiol. Chem.*, **78**, 28 (1912).

6. Anson, M. L., and Mirsky, A. E., *Proc. Soc. Exp. Biol. and Med.*, **28**, 170 (1930).
7. Clarke, H. T., *J. Biol. Chem.*, **97**, 235 (1932).
8. Küster, W., Kumpf, W., and Köppel, W., *Z. physiol. Chem.*, **171**, 114 (1927).
9. Merrill, H. B., *Ind. and Eng. Chem.*, **17**, 36 (1925).
10. Pulewka, P., *Z. physiol. Chem.*, **146**, 130 (1925).
11. Speakman, J. B., *Proc. Roy. Soc. London, Series A*, **132**, 167 (1931).
12. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 103 (1929).
13. Elek, A., and Hill, D. W., *J. Am. Chem. Soc.*, **55**, 3479 (1933).
14. Michaelis, L., and Rona, P., *Biochem. Z.*, **27**, 38 (1910).
15. Speakman, J. B., *J. Soc. Dyers and Colourists*, **41**, 172 (1925).
16. Elöd, E., and Silva, E., *Z. physik. Chem.*, **137**, 142 (1928).
17. Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, **17**, 151 (1933).
18. von Weimarn, P. P., in Alexander, J., Colloid chemistry, New York, **4**, 363 (1932).
19. Brill, R., *Ann. Chem.*, **434**, 204 (1923).
20. Meyer, K. H., and Mark, H., *Ber. chem. Ges.*, **61**, 1932 (1928).
21. Astbury, W. T., *Tr. Faraday Soc.*, **29**, 193 (1933); Fundamentals of fiber structure, London (1933).
22. Speakman, J. B., and Hirst, M. C., *Tr. Faraday Soc.*, **29**, 148 (1933).
23. Michaelis, L., and Schubert, M. P., *J. Am. Chem. Soc.*, **52**, 4418 (1930).

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THE CRYSTAL STRUCTURE OF TETRAMETHYL AMMONIUM FLUOSILICATE

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Crystals of $[(CH_3)_4N]_2SiF_6$ were prepared by slow evaporation of a solution of tetramethyl ammonium hydroxide slightly acidified with hydrofluosilicic acid. The crystals were short, rectangular prisms with corners cut by (111) faces. Goniometric measurement failed to detect any departure from cubic symmetry but since under polarized light they are optically uniaxial, they must be considered as tetragonal ($c:a = 1.00$). Laue photographs taken parallel to the optic axis possessed four-fold symmetry but no planes, indicating that the structure is isomorphous with one of the hemihedral point groups S_4, C_4 or C_4^h. Similar photographs with the X-rays traveling along the "crystallographic" a-axis showed no departure from complete four-fold symmetry. These photographs are reproduced in Figures 1 and 2.

Oscillation photographs using CuK-radiation were made about the four-fold axis, the crystallographic a-axis and [110]. All of the reflections obtained on them and on the Laue photographs can be explained in terms of a unit cell having $a_0 = 7.88$ Å, $c_0 = 11.19$Å, these axes being 45° to those of the above crystallographic description. The density, obtained by the usual flotation method was determined to be 1.42, from which computation shows that the unit cell having the dimensions given above will contain two molecules ($m = 2.06$).

A few typical reflections from a Laue photograph are listed in Table I; data from oscillation photographs are shown in Table II. Spectrometric measurements of the intensities of reflections of the $CuK-\alpha$ lines from all ($hk0$) and ($h0l$) planes having $2\theta <$ ca. 100° are recorded in Tables III and IV.

The samples used for spectrometric study were pseudocubic prisms mounted with shellac on fine glass rods, properly oriented by means of a polarizing microscope and an optical goniometer, and finally reduced to cylindrical shape. The specimens needed for the $(hk0)$ reflections could readily be set upon a basal face so that by careful manipulation very uniform cylindrical sections were obtained and the intensities from them showed very good agreement. Their averages, the averages of the reflection F's derived from them and the maximum observed deviations from these F's are listed in Table III.

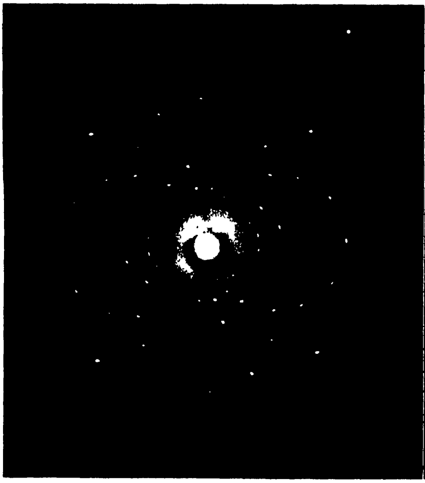

FIG. 1.

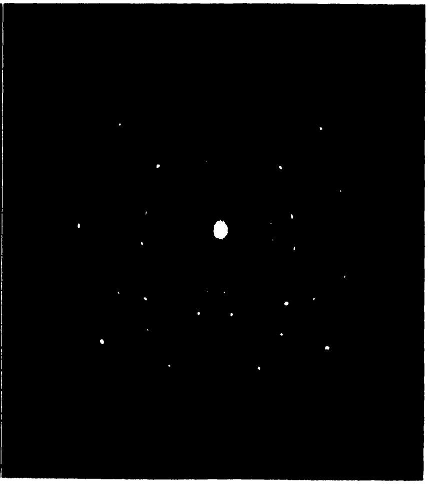

FIG. 2.

FIG. 1. A Laue photograph of $[((CH_3)_4N]_2SiF_6$ taken with the X-ray beam parallel to the optic axis, showing four-fold symmetry but no planes.

FIG. 2. A photograph similar to Figure 1, but with the X-rays traveling along the "crystallographic" a-axis.

The samples for measuring $(h0l)$ reflections were of necessity mounted upon a cube edge, which not only added much to the difficulties of manipulation, but also made it impossible to produce cylinders of uniform cross section throughout. In spite of the care taken to eliminate inequalities of surface and shape, the intensities obtained differed widely in the two specimens and in duplicate sets of readings made upon each crystal with the setting 180° from the original one. Variations as great as 50% were observed in measurements of (004), (006), (305), (501) and (107). Photographs taken before and after

TABLE I

Reflections Found on a Laue Photograph of $[(CH_3)_4N]_2SiF_6$ *Taken with X-Rays Parallel to Crystallographic a-Axis*

Indices 2 molecule unit	Estimated Intensity	$n\lambda$
107	m	.320
3,0,13	f−	.279
217	f	.300
325	f+	.497
433	f−	.405
435	ff	.298

TABLE II

Reflections Found on Oscillation Photographs of $[(CH_3)_4N]_2SiF_6$ *Taken with CuK-Radiation*

Indices 2 molecule unit	Estimated Intensity	Indices 2 molecule unit	Estimated Intensity	Indices 2 molecule unit	Estimated Intensity
Oscillation about 4-fold axis					
200	m−	420	f+	600	m
220	f	440	f	620	m
310	m−	510	f−	640	ff
330	m−	530	f	660	ff
400	ff				
Oscillation about crystallographic *a*-axis					
004	m−	114	f	2,2,10	ff
006	s−	224	ff	332	f+
116	m−	336	m−	442	ff
118	f	444	f−	0,0,12	f+
228	f+	554	ff	2,2,12	ff
448	f−	008	f+	330	m
3,3,10	ff	1,1,10	ff	440	f+
220	f+	112	m		
Oscillation about crystallographic [110]					
101	s−	105	f+	107	f+
200	s−	400	f−	008	f−
103	m−	402	m−	600	f
202	m−	006	m	109	ff
004	f+	305	f−	408	ff
301	m	206	ff	606	ff
303	ff				

spectrometric measurement showed that none of these irregularities was to be ascribed to changes in crystal orientation. The data obtained by averaging these four sets of intensities are listed in Table IV.

Large changes of intensity, resulting from small irregularities in the shape of the sample, have already been noted in a previous determination of structure in which copper radiation was used1). Together with the data of this paper they emphasize the importance of basing determinations of structure upon more than a single series of intensity data, especially when long wave length X-rays are used.

TABLE III

Intensities and Structure Factors for (hk0) Reflections

Indices	Average Intensities		F' Observed		F' Calculated
	Observed	Corrected	Average	Maximum Deviation	
110	0	0	0.0	—	−4.3
200	6464	14408	68.6	0.1	85.7
220	1534	1765	29.3	1.7	62.6
310	2684	3482	44.1	3.0	33.1
400	682	724	23.6	1.0	26.0
330	2968	3972	57.4	2.8	70.5
420	1790	2112	43.5	0.9	54.2
510	739	789	29.2	1.1	31.5
440	681	724	30.1	2.6	29.0
530	989	1080	37.6	2.7	46.2
600	1438	[1639]	47.1	0.0	51.9
620	273	280	20.1	0.5	13.1
710	0	0	0.0	—	1.3
550	0	0	0.0	—	5.8
640	367	379	24.3	1.8	25.9
730	0	0	0.0	—	5.7
800	355	366	22.8	1.3	27.3
820	*vw*	—	*vw*	—	12.1
660	409	424	22.9	2.0	20.3

The absence of planes of symmetry in Laue photographs taken parallel to the optic axis limits the choice of space groups to those isomorphous with hemihedral point groups, namely, S_4^1, S_4^2, C_4^1, C_4^2, C_4^3, C_4^4, C_4^5, C_4^6, C_{4h}^1, C_{4h}^2, C_{4h}^3, C_{4h}^4, C_{4h}^5, and C_{4h}^6. The presence of (006)

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1) R. B. Corey and R. W. G. Wyckoff, Z. Krist. **87**, 264 (1934).

TABLE IV

Intensities and Structure Factors for (h0l) Reflections

Indices	Average Intensities		F′ Observed		F′ Calculated
	Observed	Corrected	Average	Maximum Deviation	
101	8105	17997	57.9	16.8	74.2
002	0	—	0.0	—	−1.2
200	7830	14469	68.7	0.2	85.7
103	3847	4914	43.5	7.2	34.1
202	2091	2377	30.3	4.3	−46.0
004	2935	3515	40.7	12.1	63.7
301	2273	2693	37.5	9.7	−24.4
204	0	—	0.0	—	2.9
303	*vw*	—	*vw*	—	−8.4
105	2426	2786	40.6	11.0	43.3
400	550	570	20.9	2.0	26.0
402	1229	1345	33.2	4.9	−26.4
006	6392	10084	89.5	31.0	90.5
305	765	802	27.0	8.2	−27.3
206	798	845	28.3	4.2	19.5
404	0	—	0.0	—	−4.9
501	403	413	20.7	6.3	19.6
107	2508	2916	55.1	16.6	47.8
503	*vw*	—	*vw*	—	9.5
008	1075	1146	37.4	12.1	36.2
307	0	—	0.0	—	1.3
406	0	—	0.0	—	−0.1
600	1485	1639	[47.1]	0.0	51.9
208	402	412	23.4	5.5	9.8
505	*vw*	—	*vw*	—	9.9
602	0	—	0.0	—	4.9
109	283	288	20.1	6.6	19.6
604	162	163	15.7	1.9	16.5
408	*vw*	—	*vw*	—	8.0
701	290	296	21.5	2.9	24.7
507	*vw*	—	*vw*	—	19.7
309	*vw*	—	*vw*	—	−1.0
0,0,10	0	—	0.0	—	16.7
703	0	—	0.0	—	2.4
606	385	394	24.5	5.9	26.9
2,0,10	*vw*	—	*vw*	—	−7.6
705	*vw*	—	*vw*	—	19.0
1,0,11	*vw*	—	*vw*	—	16.6
800	300	305	20.8	2.9	27.3
509	0	—	0.0	—	8.4
802	0	—	0.0	—	1.8
4,0,10	0	—	0.0	—	−9.9
608	158	160	13.7	6.3	16.1
3,0,11	0	—	0.0	—	−11.1
804	0	—	0.0	—	7.4
0,0,12	732	766	30.0	10.9	40.0

excludes C_4^6 and C_{4h}^6. Of the remaining groups only S_4^2, C_4^5 and C_{4h}^5 explain the observed absence of planes (hkl) where $h+k+l$ is odd. Two molecules of $[(CH_3)_4N]_2SiF_6$ may be placed in C_{4h}^5 so as to form a structure similar to those found for other fluosilicates and the analogous chlorostannates. Each of the silicon atoms at (a), (000), $(\frac{1}{2}\frac{1}{2}\frac{1}{2})$1), may be surrounded by six fluorines, four in the special positions (h), ($uv0$) etc., and $(u+\frac{1}{2}, v+\frac{1}{2}, \frac{1}{2})$ etc., and two at (e), ($00u$), ($00\bar{u}$), $(\frac{1}{2}, \frac{1}{2}, u+\frac{1}{2})$, $(\frac{1}{2}, \frac{1}{2}, \frac{1}{2}-u)$. Each of the nitrogens at (d), $(0\frac{1}{2}\frac{1}{4})$, $(\frac{1}{2}0\frac{1}{4})$, $(0\frac{1}{2}\frac{3}{4})$, $(\frac{1}{2}0\frac{3}{4})$ may be surrounded tetrahedrally by four carbons in general positions (i).

The $Si-F$ separation in $(NH_4)_2SiF_6$ has been found2) to be 1.72 Å. For the assignment of tentative parameters the $N-C$ distance is assumed to be 1.35 Å, the fluorine radius 1.33 Å and the CH_3 radius 2.04 Å. If the ions are symmetrically arranged as in Figure 3 packing conflicts are immediately evident. Thus the distance between fluorine at ($\bar{v}u0$) and CH_3 at $(y+\frac{1}{2}, \frac{1}{2}-x, \frac{1}{2}-z)$ is 2.25 Å instead of 3.37 Å. Packing may be obtained by rotating SiF_6 in a clockwise, and $N(CH_3)_4$ in a counterclockwise direction until CH_3 at (xyz) is equidistant from the fluorines at ($uv0$) and ($v\bar{u}0$). A rotation of about $-34°$ for SiF_6 and $+12°$ for $N(CH_3)_4$ places CH_3 (A) equidistant (3.41 ± 0.02 Å)

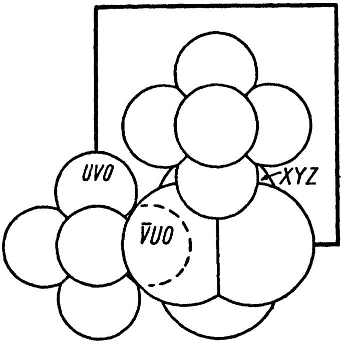

FIG. 3.

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1) R. W. G. Wyckoff, The Analytical Expression of the Results of the Theory of Space Groups, 2nd Ed. p. 82, 1930 (Washington).

2) R. M. Bozorth, J. Am. Chem. Soc. **44**, 1066 (1922).

from three fluorines (*B*, *C* and *D* of Figure 4). In this arrangement the variable parameters have the following values:

F at (h): $u = 0.18, v = 0.12$
F at (e): $\acute{u} = 0.155$
CH_3 at (i): $x = 0.14, y = 0.47, z = 0.175.$

Methyl groups in the same XY-plane just contact (4.01 Å) with each other. The distances within the ions become $Si-F = 1.73$ Å and $N-CH_3 = 1.40$ Å.

Similar packing may be obtained by turning both groups through these same angles but in the reverse directions. The structure factors of such planes as (620) calculated for this reverse rotation show it to be untenable.

In all calculations in this paper, the scattering powers[1]) of silicon are those found in silicates. Those for fluorine and nitrogen are the values obtained from alkali fluorides and ammonium chloride respectively. CH_3 was taken to have the same scattering as carbon in urea[2]). The ($hk0$) reflections calculated from a "clockwise" rotation of the SiF_6 groups, and corresponding to the parameters listed above, are given in Table III. The agreement among the more complex reflections is entirely satisfactory. The only outstanding discrepancy is found in (220) for which the observed intensity is too weak. It is doubtless to be attributed to excessive extinction and to the high absorption that prevails at small angles. A careful inspection of plots of the structure factors of ($hk0$) planes fails to reveal any changes in parameters which will lead to a general improvement.

The calculated and observed structure factors for ($h0l$) planes are shown in Table IV. The small size of the available specimens and the consequent faintness of all reflections from them made it impossible to measure quantitatively the weaker planes. Reflections marked "0·0" were not detected by the spectrometer; those labeled "*vw*" were exceedingly faint but present. Considering the impossibility of obtaining samples of uniform size and shape together with the fact that no corrections for absorption could be made, the general agreement between calculated and observed F's is as good as could be expected.

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[1]) R. W. G. Wyckoff, The Structure of Crystals, 2nd Ed. p. 100, 1931 (New York). [2]) R. W. G. Wyckoff, Z. Krist. **81**, 102 (1932).

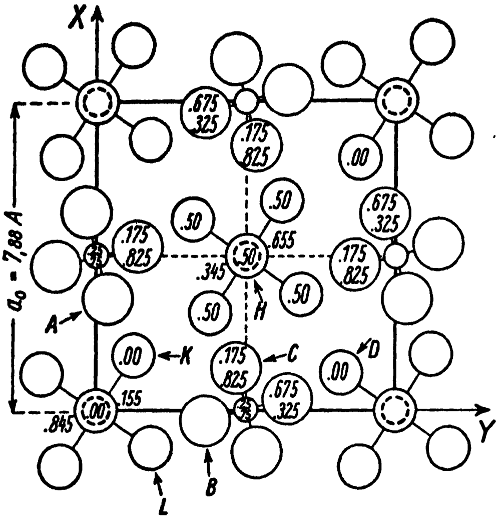

FIG. 4a. A projection upon the XY-plane of the structure found for $[(CH_3)_4N]_2SiF_6$. Dotted circles represent silicon atoms; the nitrogen and oxygen atoms and the CH_3 groups are shown by full circles of increasing size.

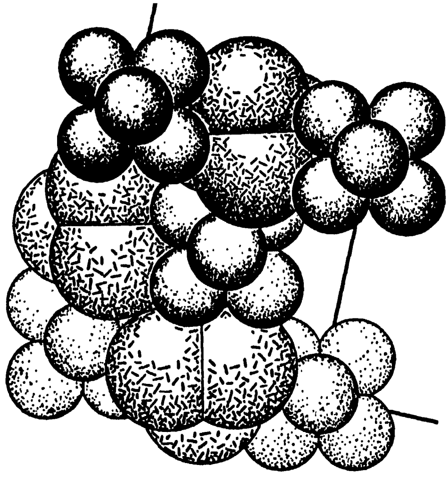

FIG. 4b. A packing drawing of 4a showing the way some of the ions fit together.

A projection of the structure upon the XY-plane is shown in Figure 4. The arrangement is a distortion of the fluorite structure already found for the cubic chloroplatinates and chlorostannates. Each methyl group contacts with one fluorine at (e) and two at (h). Thus methyl C at z $(CH_3) = 0.175$ touches fluorines H (3.43 Å), K (3.40 Å) and D (3.39 Å). Each fluorine is equidistant from four methyl groups, those at (e) being separated from each methyl by 3.43 Å and those at (h) by 3.40 ± 0.01 Å. The distance CH_3-N is 1.40 Å, in good agreement with that found in other tetramethyl ammonium compounds. The $Si-F$ separation of 1.73 Å is practically identical with that prevailing in ammonium fluosilicate (1.72 Å). It is to be noticed that the $(CH_3)_4N$ tetrahedra are in contact with each other. Thus each methyl is 4.01 Å from another methyl, both groups being located in the same XY-plane (see A and C, Figure 4).

Many of the calculations of this paper were made by K. Pestrecov and A. A. Murtland.

SUMMARY

Laue and oscillation photographs show that the tetragonal unit cell of $[(CH_3)_4N]_2SiF_6$ contains two molecules and has the dimensions $a_0 = 7.88$ Å, $c_0 = 11.19$ Å. The space group is C_{4h}^5 with silicon at (a), fluorine at (e) and (h), nitrogen at (d) and methyl occupying general positions (i). The atomic parameters were determined from spectrometric measurements of reflections from all ($hk0$) and ($h0l$) planes for which $2\theta <$ ca. 100°. Best agreement was obtained when the following parameters were assigned to fluorine and methyl:

F at (h): $u = 0.18$, $v = 0.12$,
F at (e): $u' = 0.155$,
CH_3 at (i): $x = 0.14$, $y = 0.47$, $z = 0.175$.

The structure is a distorted fluorite arrangement of SiF_6 groups and $(CH_3)_4N$ tetrahedra. Interatomic distances agree well with those previously determined in other compounds.

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CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

IX. THE SPECIFICITY OF ANTIGENS PREPARED BY COMBINING THE *p*-AMINOPHENOL GLYCOSIDES OF DISACCHARIDES WITH PROTEIN

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As a result of the work of numerous investigators a striking advance has been made during the past decade in our knowledge of the chemical and immunological properties of that unique class of naturally occurring substances, the specific bacterial polysaccharides. Complex carbohydrates possessing immunologically specific properties have been found widely distributed in many varieties of different microorganisms. The significance of these findings lies in the fact that these specific components, having in common the chemical properties of polysaccharides, are known to function as determinant substances in many of the specific reactions of bacterial infection and immunity. The fact that these carbohydrates, so closely related in chemical properties, remain sharply differentiated in biological specificity may eventually be explained on the basis of certain subtle differences in the chemical structure of the molecules that constitute these substances. Possible differences in stereochemical structure and in the position of intermolecular linkages between the individual sugars comprising the polysaccharides are manifold. Indeed, it is not unlikely that structural differences of this character account for the wide variation in the immunological specificity exhibited by carbohydrates of diverse origin.

It is already known that differences in the stereochemical structure of the carbohydrate radical in artifically compounded hexoside-protein antigens influence their immunological specificity (1). It has been shown that the azophenol glycosides of glucose and galactose, irrespective of the protein with which they are combined, give rise in the animal body to the formation of antibodies which are distinct and specific for the carbohydrate radical in the antigenic complex. Although the difference in chemical structure between the glycosides of glucose and galactose is confined to an interchange of the H and OH groups on the fourth carbon atom of the hexose radical, yet this slight alteration in chemical configuration suffices to determine the biological specificity of antigens containing these hexosides. If, on the other hand, the structural differences in monosaccharides be confined to the carbon atom bearing the aglucon, as in antigens containing α- and β-azophenol glucosides, then the immunological reactions of these two derivatives of glucose, though predominantly type-specific, show a certain degree of crossing, presumably attributable to the identity in structure of the five terminal carbon atoms of both glucosides (2).

Thus it has been established experimentally that the stereochemical configuration of the hexose radical has an important influence in determining the biological specificity of artificial antigens containing simple monosaccharides. Whether stereochemical changes in the hexose constituents of more complex saccharides exert a similar influence, and whether the position of linkage of hexose to hexose affects the course of antibody response, are questions of importance in elucidating the factors which govern the specificity of the reactive polysaccharides of bacterial origin.

The disaccharides are substances of known chemical constitutions, and hence are excellent derivatives to use in studying the influence of changes in intermolecular linkage and stereochemical structure on biological specificity. For this purpose, therefore, the *p*-aminophenol glycosides of lactose, gentiobiose, cellobiose, and maltose have been synthesized (3). The diazonium derivatives of these glycosides have been combined with protein and the resulting conjoined disaccharide-proteins have been utilized as antigens in the preparation of immune rabbit sera. The present paper is an account of the immunological properties of these artifically conjugated antigens. For purposes of comparison there are also included the serological properties of antigens containing monosaccharide derivatives prepared by combining with the same protein the *p*-aminophenol glycosides of α- and β-glucose and of β-galactose.

EXPERIMENTAL

1. Methods.—The methods of preparing the mono- and disaccharide immunizing antigens used in the present study differ in no essential respect from those previously described (2 *b*). The method of intravenous immunization of rabbits, and the technique of the precipitin and inhibition tests, are the same as those employed in earlier studies. The *immunizing* antigens were prepared by combining each of the carbohydrate derivatives with the globulin of normal horse serum. In order to avoid the reactions of a common protein, the *test* antigens were similarly prepared by combining the same glycosides to the protein of chicken serum.

For purposes of brevity the *p*-aminophenol glycosides of the different sugars will be frequently referred to throughout the text and tables by the following abbreviations: α = α-glucoside; β = β-glucoside; Ga = β-galactoside; C = β-cellobioside; M = β-maltoside; Ge = β-gentiobioside; and L = β-lactoside. The immunizing antigens prepared by combining these glycosides with globulin are referred to as α-globulin, C-globulin, etc., and the corresponding antisera respectively as α-antiserum, C-antiserum, etc. The test antigens, prepared by combining the glycosides with chicken serum protein are designated as α-test antigen, C-test antigen, etc.

I. Specific Precipitin Tests

1. Homologous Precipitin Reactions of Mono- and Disaccharide Antisera.—The sera of rabbits immunized respectively with antigens containing the three monosaccharide and the four disaccharide glycosides combined with horse serum globulin were first studied for the presence of homologous precipitins. Test antigens containing the same glycosides combined with the protein of chicken serum were used in the precipitation tests. The results of these tests are given in Table I.

From the results presented in Table I it is seen that antigens containing either mono- or disaccharide radicals give rise in each instance to immune bodies which react with the same carbohydrate derivative irrespective of the protein to which it is attached. The glycoside-proteins function as effective antigens in the animal body, and it is suggested that the large number of polar (OH) groups in the carbohydrate radical may account for this fact.

2. Heterologous Precipitin Reactions of the Disaccharide Antisera.—In order to ascertain whether the disaccharide antisera cross-react with test antigens containing the other disaccharides, each serum was tested for the presence of heterologous precipitins.

The results of the cross-precipitin tests are summarized in Table II

From the results given in Table II, it can be seen that an antiserum prepared by immunization with C-globulin contains antibodies which precipitate not only the homologous C-test antigen, but the heterologous Ge-test antigen as well. Similarly the antiserum to Ge-globulin contains precipitins for the homologous test antigen and cross-reacts with the heterologous C-test antigen. Neither of these antisera, however, reacts to any appreciable extent with test antigens containing M or L. The M-antiserum, on the other hand, precipitates not only M- but also Ge- and C-test antigens; the M-antiserum does not react with test antigen containing L. The L-globulin antiserum precipitates both the homologous and the heterologous C-test antigen; however, it does not react with test antigens in which M or Ge is present.

TABLE I

*Homologous Precipitin Reactions of Mono- and Disaccharide Antisera**

Antisera prepared by immunization with:	Test antigens†	Final dilution of test antigens			
		1:5,000	1:10,000	1:20,000	1:40,000
α-Globulin	α Chicken serum	+++±	+++±	++±	++
β "	β " "	++++	++++	+++	+++
Ga "	Ga " "	+++±	+++	++±	++
C "	C " "	++++	++++	+++±	+++
M "	M " "	++++	++++	++++	+++
Ge "	Ge " "	+++±	+++	+++	++±
L "	L " "	++++	+++±	+++	++±

++++ = complete precipitation with compact disk formation.

± = faint turbidity when read with artificial illumination against dark background.

0 = no precipitation.

* The immune sera were in all instances used in constant amounts of 0.2 cc. A dilution of serum in the proportion of two parts of serum to three parts of salt solution was prepared, and 0.5 cc. of this dilution, containing 0.2 cc. of the original serum, was added to 0.5 cc. of the varying dilutions of test antigens, in Tables I to IV.

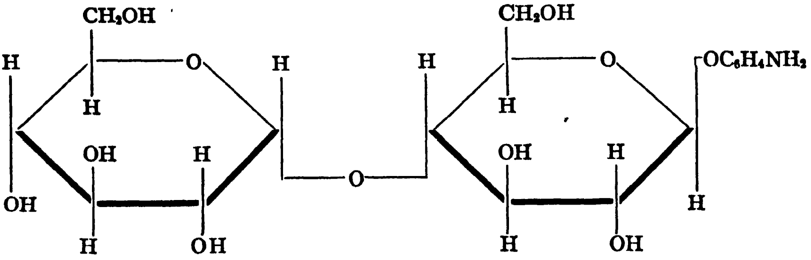
† Test antigens were prepared by combining the respective glycosides with the protein of chicken serum.

The cross-precipitin reactions of these four disaccharide antisera appear at first difficult of interpretation, but further consideration of the stereochemical structures of the four glycosides affords an insight into the complexities of the cross-reactions. Each of the disaccharide glycosides has in common a large chemical grouping; namely, a glucose molecule substituted by the aglucon—$C_6H_4N=N$— combined in glucosidic union with the reducing group of the hexose. In the case of the gentiobioside and cellobioside, each glycoside contains a terminal β-glucose molecule substituted in positions 6 and 4 respectively of the glucose molecule bearing the aglucon. The maltoside, on the other hand, has an α-glucose molecule substituted in position 4, whereas the lactoside has a β-galactose molecule in this same position. The constitutional and configurational relationships of these four glycosides may best be understood by the following graphic formulae:

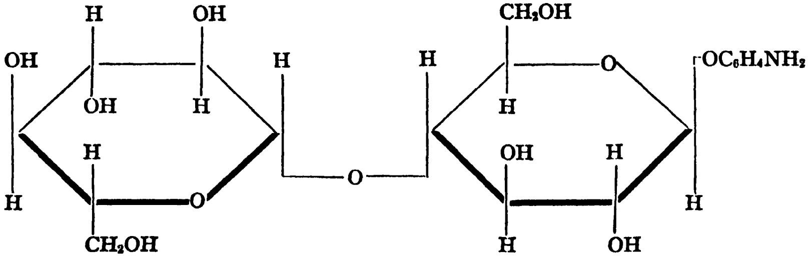
TABLE II

Heterologous Precipitin Reactions of Disaccharide Antisera

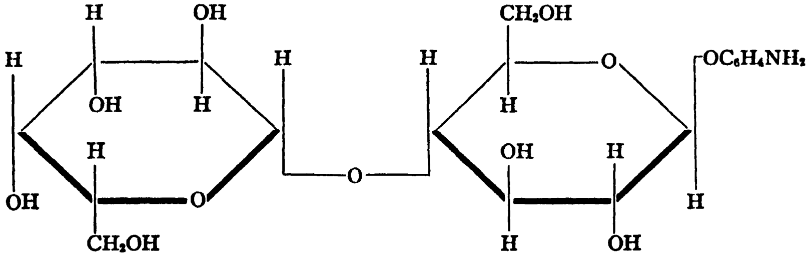
Antisera prepared by immunization with:	Test antigens	Final dilution of test antigens		
		1:5,000	1:10,000	1:20,000
C-Globulin	C Chicken serum	++++	++++	+++±
	M “ “	±	±	±
	Ge “ “	++±	++	+±
	L “ “	±	±	±
M-Globulin	C Chicken serum	+++	++±	+±
	M “ “	++++	++++	+++±
	Ge “ “	++±	+±	+
	L “ “	±	±	±
Ge-Globulin	C Chicken serum	+++	+++	++
	M “ “	±	±	±
	Ge “ “	++++	+++±	++±
	L “ “	±	±	±
L-Globulin	C Chicken serum	++±	++±	++
	M “ “	±	±	±
	Ge “ “	±	±	±
	L “ “	+++±	+++	+++



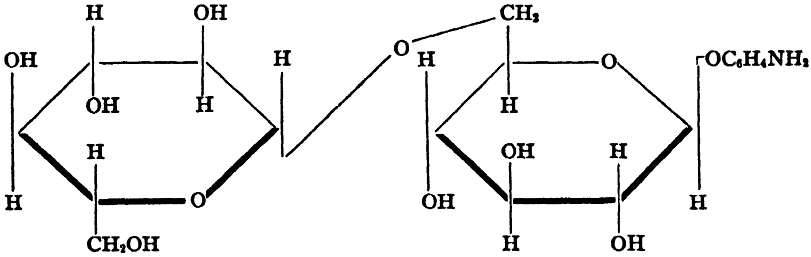
p-Aminophenol *β*-maltoside



p-Aminophenol *β*-cellobioside



p-Aminophenol *β*-lactoside



p-Aminophenol *β*-gentiobioside

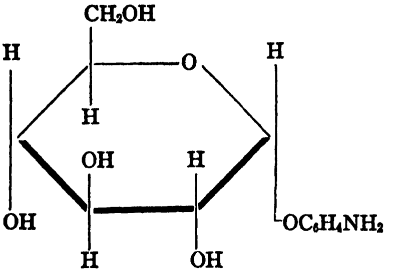
From the graphic formulae it can be seen that the carbohydrate radicals of the cellobioside and gentiobioside are each composed of two units of glucose combined in glucosidic union. In the case of the cellobioside, the union of hexose molecules is on the fourth carbon atom of the glucose molecule bearing the aglucon; in the gentiobioside this union is on carbon atom 6. Apparently as a result of the similarity in structure, and because of the β configuration of the terminal glucose molecule common to both glycosides, antigens containing either C or Ge show reciprocal cross-reactions in the corresponding antisera. The position of linkage of hexose to hexose is apparently less important in determining specificity than is the configuration of the terminal hexose. The configuration of the individual carbon atoms of the terminal hexose is also an important factor in determining specificity, since an antigen containing L does not react in C antiserum despite the fact that the structures of C and L are identical save in respect to the spatial arrangement of the H and OH groups on the fourth carbon atom of the terminal hexose.

Although the M-test antigen fails to precipitate either in C- or Ge-antiserum, it is a striking fact that both C- and Ge-test antigens precipitate in an antiserum prepared by immunization with maltoside-globulin. When one considers these cross-reactions with reference to the chemical structure of the three glycosides, it appears that the specificity of the immunological response is more sharply defined when the terminal hexose in the antigenic molecule has the β rather than the α configuration. The reason for this is not known.

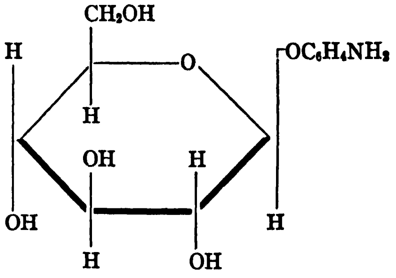
It has been previously pointed out that the four glycosides, M, C, Ge, and L, each possess in common a large molecular grouping; namely, a glucose molecule bearing the aglucon—C_6H_4—N=N—. In view of this fact one might expect the corresponding antisera to show marked cross-reactions with test antigens containing any one of the heterologous glycosides. That this is not the case, however, is evident from the results of the precipitin tests given in Table II. It may be concluded, therefore, that the large grouping common to all the disaccharide antigens exerts but little influence on serological crossing and that in those instances in which this phenomenon occurs the reactions are determined primarily by similarities in the configuration of the terminal hexose of the glycosides.

One striking exception, however, has been encountered in the case of the lactoside, in which the terminal hexose is β-galactose. An antiserum to the lactoside would, on the basis of the preceding hypothesis, not be expected to precipitate an antigen having a terminal β-glucose molecule. However, it has been shown that L-antiserum contains precipitins reactive with C-test antigen, although the reaction is not reciprocal. There is at present no adequate explanation for this phenomenon. It may be pointed out that Landsteiner and Lampl (4) encountered a series of non-reciprocal reactions in the course of investigations on the specificity of antisera to *o*-aminobenzene sulfonic acid, and *o*-aminobenzoic acid. Similarly Heidelberger and Kendall (5) have described in detail an analogous series of non-reciprocal precipitin reactions employing antisera prepared by immunization of rabbits with R-salt-azo-benzidine-azo-crystalline egg albumin and native crystalline egg albumin.

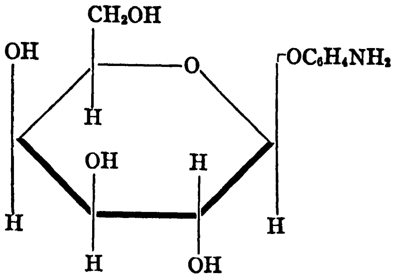
3. Heterologous Precipitin Reactions of Disaccharide Antigens in Monosaccharide Antisera.—In order to gain further insight into the factors which determine the serological specificity of disaccharide antigens, the latter were tested in the immune sera prepared by the immunization of rabbits with antigens containing the azophenol glycosides of α- and β-glucose and β-galactose combined with horse serum globulin. The structural relationship of the hexosides from which the corresponding monosaccharide antigens were prepared is represented by the following graphic formulae:



p-Aminophenol α-glucoside



p-Aminophenol β-glucoside



p-Aminophenol *β*-galactoside

From the above formulae it can be seen that the *α*- and *β*-glucosides differ from one another in the stereochemical arrangement of the carbon atom bearing the aglucon. The remaining five carbon atoms of each hexoside have the same stereochemical pattern. The *β*-galactoside differs from the *β*-glucoside in that in each instance the H and OH groups on the fourth carbon atom are interchanged. Before turning to the results of the precipitin tests of the disaccharide test antigens in the monosaccharide antisera, it must be recalled that the terminal hexose of both the cellobioside and the gentiobioside is *β*-glucose, while the terminal molecule of the maltoside is *α*-glucose, and of the lactoside, *β*-galactose.

From the results of the precipitin reactions of disaccharide antigens in monosaccharide antisera (Table III) it is interesting to observe that only the test antigen containing the maltoside, the terminal hexose of which is *α*-glucose, reacts in *α*-antiserum. The two test antigens containing the cellobioside and gentiobioside, both of which have terminal *β*-glucose radicals, react only in *β*-antiserum, and finally the lactoside test antigen, the terminal hexose radical of which is galactose, reacts only in *β*-galactoside antiserum. The results of these serological tests support the view that the stereochemical configuration of the terminal hexose in disaccharide antigens is the dominant factor in determining their serological crossing in monosaccharide antisera.

4. Precipitin Reactions of Monosaccharide Antigens in Disaccharide Antisera.—In order to determine whether the monosaccharide test antigens react with the disaccharide antisera, the latter were tested for the presence of precipitins reactive with *α*-, *β*- and Ga-test antigens. The results of the precipitin tests are given in Table IV. From the results given in Table IV it may be seen that only the α-test antigen reacts in M-antiserum, whereas the β-test antigen reacts in both C- and in Ge-antiserum as well as in M-antiserum. It has been previously shown that both C- and Ge-test antigens, the carbohydrate radicals of which both contain a terminal β-glucose molecule, react in

TABLE III

Precipitin Reactions of Disaccharide Antigens in Monosaccharide Antisera

Antisera prepared by immunization with:	Test antigens	Final dilution of test antigens		
		1:5,000	1:10,000	1:20,000
α-Globulin	α Chicken serum	+++	++±	++±
	C " "	±	0	0
	M " "	++±	++±	++
	L " "	0	0	0
	Ge " "	0	0	0
β-Globulin	β Chicken serum	++++	++++	+++±
	C " "	+++	++±	++
	M " "	±	0	0
	L " "	0	0	0
	Ge " "	+++	+++	++
Ga-Globulin	Ga Chicken serum	+++	++±	++
	C " "	0	0	0
	M " "	0	0	0
	L " "	+±	++	++
	Ge " "	0	0	0

TABLE IV

Precipitin Reactions of Monosaccharide Antigens in Disaccharide Antisera

Antisera prepared by immunization with:	Final dilution of α chicken serum antigen			Final dilution of β chicken serum antigen			Final dilution of Ga chicken serum antigen		
	1:5,000	1:10,000	1:20,000	1:5,000	1:10,000	1:20,000	1:5,000	1:10,000	1:20,000
C-Globulin..........	±	±	±	++±	++	++	0	0	0
M "	+++	+++	++	+++	++±	++	0	0	0
L "	0	0	0	0	0	0	++	+±	+
Ge "	±	±	±	++±	++±	++	0	0	0

M-antiserum. It is not surprising therefore, that an antigen containing a monosaccharide radical having the β-glucose configuration also reacts in the same serum. It is also evident from Table IV that the Ga-test antigen, which possesses a terminal β-galactose molecule, reacts only in L-antiserum. These serological findings again emphasize the fact that the configuration of the terminal hexose is a dominant factor in determining the specificity of antibodies induced by disaccharide antigens.

TABLE V

*Inhibition of Precipitins in Cellobioside-Globulin Antiserum by Homologous and Heterologous Glycosides**

Inhibiting glycoside	Test antigens (final dilution 1:5,000)		
	C	Ge	β
None	++++	++	++±
α	++++	+±	++
β	++++	0	0
Ga	++++	++	++
C	0	0	0
M	+++±	+±	++
L	+++±	+±	++±
Ge	+++±	0	0

* The technique of the specific inhibition tests given in Tables V to VIII was as follows: 0.3 cc. of a 0.1 M solution of the different *p*-aminophenol glycosides was added to 0.2 cc. samples of immune serum. The mixtures were incubated for 2 hours at 37°C. and to them was then added 0.5 cc. of a 1:2,500 dilution of the reactive test antigens. The tubes were again incubated for 2 hours at 37°C. and the final readings were recorded after the tubes had stood overnight in the ice box.

II. Specific Inhibition Tests

1. Cellobioside-Globulin Antiserum.—The selective specificity of the precipitins in cellobioside-globulin antiserum for test antigens containing the cellobioside, the gentiobioside, and the β-glucoside can be seen from the results of the specific inhibition tests given in Table V.

An analysis of the data presented in Table V shows that the specific reaction between C-antiserum and its homologous test antigen can be inhibited only by C, whereas the cross-reaction of Ge-test antigen in C-antiserum is inhibited by C, Ge, and by β. These three glycosides each have a terminal *β*-glucose radical. It must be pointed out that the heterologous reactions, though somewhat diminished, are not completely inhibited by *α* or by M, both of which are glycosides having a terminal *α*-glucose radical. Likewise the cross-reaction between C-antiserum and Ge-test antigen is not inhibited by L or by Ga, both of which contain a terminal *β*-galactose radical. Similarly, the cross-reaction between *β*-test antigen and C-antiserum is inhibited by C, Ge, and by *β*, but by none of the other glycosides.

The results of the specific inhibition tests emphasize the fact that an antigen containing a cellobiose radical gives rise to antibodies which are specific for this particular carbohydrate grouping. Since the reaction between C-antigen and homologous antiserum cannot be inhibited by the glycoside of gentiobiose, a disaccharide which is isomeric with cellobiose, it may be concluded that the position of the biose linkage is important in determining the biological specificity of disaccharide antigens. From the fact that the heterologous reactions of *β*- and Ge-test antigens in C-antiserum can be inhibited only by glycosides containing a terminal *β*-glucose molecule, it may be further concluded that the specificity of the serological cross-reactions is determined primarily by the configuration of the terminal hexose residue of the cellobioside.

TABLE VI

Inhibition of Precipitins in Gentiobioside-Globulin Antiserum by Homologous and Heterologous Glycosides

Inhibiting glycoside	Test antigens (final dilution 1:5,000)		
	Ge	C	*β*
None	+++	+±	++±
α	++±	+±	++
β	++±	0	0
Ga	++±	+±	++
C	++±	0	0
M	++±	+±	++
L	++±	+±	++
Ge	0	0	0

2. Gentiobioside-Globulin Antiserum.—The results of the specific inhibition of precipitins in gentiobioside-globulin antiserum by the homologous and heterologous glycosides are summarized in Table VI. From the results presented in Table VI it can be seen that only the gentiobioside inhibits the reaction between Ge-antiserum and homologous test antigen. The fact that the homologous reaction cannot be inhibited by the cellobioside, a derivative which is identical with the gentiobioside in structure except for the position of the biose junction, further substantiates the importance of the position of intermolecular linkage in determining the specificity of disaccharide antigens. As in the case of the cellobioside antiserum, the heterologous precipitin reactions of Ge-antiserum are in each instance inhibited by glycosides containing a terminal β-glucose molecule, and not by those having an α-glucose or β-galactose configuration.

TABLE VII

Inhibition of Precipitins in Maltoside-Globulin Antiserum by Homologous and Heterologous Glycosides

Inhibiting glycoside	Test antigens (final dilution 1:5,000)				
	M	α	β	C	Ge
None	++++	++±	+++	++±	++±
α	+++±	0	0	0	0
β	+++±	++±	0	0	0
Ga	+++±	++±	+++	++	+±
C	+++±	++±	±	0	0
M	0	0	0	0	0
L	+++±	++±	+++	++	+±
Ge	+++±	++±	0	0	0

3. Maltoside-Globulin Antiserum.—It will be recalled that an antiserum prepared by immunization with an antigen containing azophenol maltoside cross-reacts with test antigens in which the terminal hexose residue is either α- or β-glucose, but does not react with antigens in which the terminal hexose is β-galactose. The results of the specific inhibition of precipitins in M-antiserum by the different glycosides are given in Table VII. From the results of the specific inhibition tests given in Table VII it can again be seen that precipitation of M-test antigen in homologous antiserum is inhibited only by the maltoside. The failure of the other glycosides to inhibit the homologous precipitin reaction indicates that the maltoside antigen gives rise to immune bodies specific for the disaccharide radical in question.

It has previously been pointed out that the terminal hexose of maltose is α-glucose. The configurational relationship of the six carbon atoms of this portion of the maltoside is in all respects identical with that of the simple monosaccharide derivative, α-glucoside. On the basis of this configurational identity, and in view of the fact that the specificity of the cross-precipitins elicited by antigens containing a disaccharide radical is determined primarily by the terminal hexose, the precipitation of azophenol α-glucoside in M-antiserum may be considered as approaching an homologous reaction. It is therefore to be anticipated that this reaction would be inhibited only by those glycosides possessing an α-glucose configuration. That this is indeed the case may be seen by referring to Table VII, the results of which show clearly that neither B, Ge, nor C inhibit the reaction of α-test antigen in M-antiserum. It may be concluded that the configuration of the terminal hexose of the M-immunizing antigen so orients the specificity of the antibodies induced that the latter, as evidenced by the inhibition tests, remain predominantly specific for the α-glucose configuration.

The cross-reactions of M-antiserum with the heterologous test antigens β, C, and Ge can be understood by comparing the similarity in chemical constitution of the three glycosides with that of the homologous maltoside. In each glycoside the terminal hexose is glucose. In the maltoside this hexose has the α-configuration, whereas in the β-glucoside, the cellobioside, and the gentiobioside, the terminal hexose is β-glucose. In all of these glycosides the five end carbon atoms are identical in stereochemical structure. This identity in structure may explain the cross-reactions of test antigens containing the terminal β-glucose configuration in M-antiserum. Similarly, it might be expected that test-antigens containing a terminal α-glucose molecule would reciprocally react in the antisera prepared by immunization with antigens containing a terminal β-glucose configuration. It has already been pointed out, however (*cf.* Table II), that in general this is not the case, although certain C- and Ge-antisera have been obtained which show a slight degree of crossing with both α- and M-test antigens, when the latter are used in high concentration. It appears, therefore, that disaccharide antigens containing a terminal β-glucose molecule give rise to antibodies which in general cross-react with test-antigens having a β-glucose molecule and not with those in which the terminal hexose has the α configuration. This fact appears to be true only for antigens containing the azophenol glycosides of the β-disaccharides and not for corresponding glucoside of the monosaccharide, β-glucose. The reason underlying this unusual phenomenon cannot as yet be defined with certainty. By reference to molecular models of the two disaccharides, maltose and cellobiose, it is at once apparent that the spatial relationship of the polar groups of the two hexoses is in each instance different. This difference in the orientation of these groups may modify, or even mask, the influence which the individual polar groups might otherwise exert upon the specificity of disaccharide antigen containing two hexose molecules.

It has previously been pointed out that the cross-reactions of β-, C-, and Ge-test antigens in M-antiserum may be attributed to the identity in configuration of the five end carbon atoms of each of these glycosides. It might be expected, therefore, that any glycoside having this common configuration would inhibit the reaction of β-, C-, or Ge-test antigens in M-antiserum. Indeed, this has proved to be the case, for it may be seen from Table VII that the cross-reactions of the maltoside-globulin antiserum with any one of the heterologous test antigens containing a terminal β-glucose molecule are inhibited by any one of the *p*-aminophenol glycosides, in which the terminal hexose molecule is glucose, irrespective of its configuration. However, when the configuration of one of the five terminal polar groups is altered, as in the case of the galactoside or lactoside, the latter glycosides fail to inhibit the cross-reactions between M-antiserum and test antigens containing a terminal glucose molecule.

4. Lactoside-Globulin Antiserum.—The selective specificity of the antibodies in lactoside antiserum may be seen from the results of the inhibition tests given in Table VIII.

From data presented in Table VIII, it is seen that the antigen containing the lactoside gives rise to immune bodies which are specific, since the precipitation of L-test antigen in homologous antiserum can be inhibited only by the homologous glycoside. The cross-precipitation of Ga-test antigen in L-antiserum is likewise inhibited only by the glycoside of lactose and galactose. This observation once more confirms the view that the terminal hexose of disaccharide antigens exerts a dominant influence in determining serological crossing. On the basis of this concept, it would be expected that L-antiserum would react only with test antigens containing L and Ga. However, it has already been seen (Table II) that C-test antigen reacts in L-antiserum despite the fact that the terminal hexose of the cellobioside is glucose, while that of the lactoside is galactose. The only portion of the cellobioside and lactoside which is identical in configuration is therefore the glucose molecule bearing the aglucon. The serological activity of C-test antigen in L-antiserum might be ascribed to this common grouping. It must be remembered, however, that this grouping is also common to the maltoside and gentiobioside, yet antigens containing the latter glycosides fail to react in L-antiserum. Moreover the two glycosides, Ge and M, do not inhibit the cross-reaction of C-test antigen in L-antiserum. At present there is no adequate explanation for this unusual cross-precipitin reaction, nor do the results of the specific inhibition tests throw any light on the situation. The question must await further investigation before a definite explanation can be given.

TABLE VIII

Inhibition of Precipitins in Lactoside-Globulin Antiserum by Homologous and Heterologous Glycosides

Inhibiting glycoside	Test antigens (final dilution 1:5,000)		
	L	Ga	C
None	+++	+±	++
α	+++	+±	+±
β	+++	+±	+±
Ga	+++	0	+±
C	+++	+±	0
M	+++	+±	+±
L	0	0	0
Ge	+++	+±	+±

DISCUSSION

In attempting to understand the intricate serological relationships between antigens containing the four disaccharides, gentiobiose, cellobiose, lactose, and maltose, it is necessary to have clearly in mind a picture of the stereochemical structure of each disaccharide. The reducing disaccharides, maltose, gentiobiose, lactose, and cellobiose, are compounds of two hexose molecules joined in glycosidic union through the reducing group of one of the monosaccharide constituents. The point of attachment of one hexose to the other, and the nature of the linkage (*i.e.* whether the terminal hexose has an α or β configuration) have been carefully ascertained in the case of the four disaccharides in question, and as a result their chemical structures are fully comprehended. With an exact knowledge of their structures, the disaccharides become excellent substances for study in correlating changes in chemical constitution with differences in biological specificity.

The conversion of these four disaccharides into their corresponding *p*-aminophenol glycosides (3) is without doubt accompanied by no change in the lactal ring structure of the hexose units. The glycosides may be regarded as built up from two units of hexoses in pyranoid form, with the remaining reducing group replaced by the aglucon —$C_6H_4NH_2$. The three glycosides of maltose, cellobiose, and lactose, have a common structure and are distinguishable only in that each has a different configuration. The gentiobioside, on the other hand, differs structurally from its isomers in that the point of attachment of the terminal β-glucose molecule is on the sixth carbon atom of the glucose molecule bearing the aglucon. By referring to the graphic model formulae these structural and configurational relationships may be clearly seen.

From the results of the specific inhibition tests and the heterologous precipitin reactions it may be seen that these structural and configurational relationships have a distinct and definite influence in determining the immunological specificity of antigens containing these disaccharides. The present study emphasizes the fact that the specificity of the serological cross-reactions of disaccharide antigens is determined by the configuration of the terminal hexose molecule. In a study of the immunological properties of peptides, Landsteiner and van der Scheer (6) have shown that the specificity of antigens containing amino benzoylated peptides depends primarily upon the structure of the terminal amino acid, and to a less degree upon the other amino acids in the peptide chain.

Although each of the disaccharide antigens contains in common an azophenol β-glucoside radical, this second hexoside grouping does not appear to be of importance in determining serological crossing; however, its presence does confer upon each antigen, certain specific properties. The rôle of this common hexose molecule bearing the aglucon is demonstrated by the fact that the homologous reactions between disaccharide test antigens and the corresponding antisera are not inhibited by simple hexosides conforming in configuration and structure to the terminal hexose molecule of the disaccharide. It must be borne in mind, therefore, that the individual specificity of disaccharide antigens is dependent upon the molecular pattern of the glycoside radical as a whole. Thus it has been shown that antigens containing glycosides as closely related as are the gentiobioside and cellobioside, may be specifically and sharply differentiated serologically by means of inhibition reactions.

From a comparison of the immunological properties of the different disaccharide antigens, it is evident that when the terminal hexose has the β configuration the specificity of the antibody response is more sharply defined than when this configuration is of the α type, for it has been shown that although C- and Ge-test antigens react in M-antiserum, the reverse does not hold true.

A striking analogy to the lack of reciprocal cross-reactions is found in the case of the capsular polysaccharides of Pneumococcus Types III and VIII. It has been observed that the specific polysaccharide of Type VIII Pneumococcus reacts both in Type VIII and Type III antipneumococcus serum, whereas the Type III carbohydrate reacts only in the homologous antiserum.

SUMMARY

The results of the present study indicate that by means of serological reactions it is possible to differentiate selectively the *p*-aminophenol glycosides of maltose, cellobiose, gentiobiose, and lactose. The immunological specificity of disaccharide-protein antigens prepared from these derivatives, irrespective of the nature of the conjoined protein, is determined by (1) the glycoside molecule as a whole, (2) the configuration of the terminal hexose molecule, and (3) the position of linkage of the two hexose units in the carbohydrate radical. The specificity of the antibodies induced by the disaccharide antigens appears to be more sharply defined when the configuration of the terminal hexose is of the β rather than of the α type.

BIBLIOGRAPHY

1. (*a*) Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1929, **50**, 521. (*b*) Avery, O. T., and Goebel, W. F., *J. Exp. Med.*, 1929, **50**, 533.
2. (*a*) Goebel, W. F., Babers, F. H., and Avery, O. T., *J. Exp. Med.*, 1932, **55**, 761. (*b*) Avery, O. T., Goebel, W. F., and Babers, F. H., *J. Exp. Med.*, 1932, **55**, 769.
3. Babers, F. H., and Goebel, W. F., *J. Biol. Chem.*, 1934, **105**, 473.
4. Landsteiner, K., and Lampl, H., *Biochem. Z.*, 1918, **86**, 343.
5. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1934, **59**, 519.
6. Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1932, **55**, 781; 1934, **59**, 769.

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THE EFFECT OF ANTECEDENT INFECTION AND IMMUNIZATION WITH STREPTOCOCCI UPON THE REACTIVITY OF RABBITS TO HORSE SERUM

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Tissue reactivity may be altered by infection not only in a specific sense to substances derived from the causative agent, but also non-specifically to unrelated antigenic materials. Specific alterations afford the basis for clinically useful cutaneous reactions in such diseases as tuberculosis, pneumonia, trichophytosis, lymphogranuloma inguinale, and tularemia. On the other hand, non-specific changes in reactivity are involved in fewer clinical demonstrations. Changes in the degree of capillary permeability (1) and in the character of cantharidin blister fluids (2) have been ascribed to alterations in cellular reactivity incident to certain infections.

Non-specific changes in reactivity, although well recognized, have not been studied extensively, and their influence in determining the ultimate response of the body to the introduction of foreign proteins, or of infectious agents seems worthy of additional investigation.

Most observations of this character have been made in experimentally infected animals, and many deal with the effect of tuberculosis upon antigenic responses. Dienes (3) noted that injections of egg white into tuberculous lesions in guinea pigs induced a tuberculin type of skin sensitivity to this substance. Lewis and Loomis (4) observed increased formation of hemolytic amboceptor and agglutinins against *B. typhosus* following injections of the appropriate antigens into tuberculous guinea pigs. Under similar conditions of infections Selter (5) demonstrated cutaneous hypersensitivity to extracts of colon bacilli. Experiments in tuberculous rabbits dealing only with the rate of production of hemolytic amboceptor have been reported by several observers (4, 6, 7) with highly inconstant or negative results. Certain other chronic infections likewise influence reactivity non-specifically. For example, Lewis and Loomis (8) found accelerated formation of hemolytic amboceptor in guinea pigs infected with streptococci or *B. abortus.* Blumenberg (9) demonstrated tuberculin sensitivity in rabbits when severe chronic paratyphoid infections were present. Schroeder (10) observed increased hemolytic amboceptor production following sheep red blood cell injections into stock rabbits suffering from spontaneous abscesses; but experimental infections with staphylococcus or *B. pyocyaneus* did not produce this effect, possibly because the artificial infections were not chronic in character. Wessely (11) in 1911 described an "anaphylactic type" of keratitis that developed after an interval of 2 weeks in a majority of normal rabbits receiving intracorneal injections of horse serum. This reaction was subsequently studied, particularly in luetic rabbits, by a number of investigators with conflicting results (12–16). Among these, vom Hofe and Krantz (16), and Löwenstein (12), found that keratitis developed with greater frequency and intensity in syphilitic rabbits.

It appears, therefore, that certain infections, particularly those chronic in nature, accelerate non-specific responses. This phenomenon, however, has been observed only in the stage of active infection, suggesting that the reaction may be due in large part to transient physiological effects of fever and toxemia rather than to factors exclusively conditioned by infection. That an infection may alter *subsequent* response, and that this alteration may be of considerable significance is suggested by the fact that non-specific changes of reactivity of decisive influence have been induced experimentally by preliminary injection of antigenic materials.

Semsroth and Koch (17) found that preliminary treatment of guinea pigs with horse serum and of rabbits with streptococcal vaccines or casein markedly altered the reactivity of the endocardium; this was demonstrable upon the subsequent establishment of a staphylococcal infection. Their experience corresponded with that of others to whom they refer (especially Siegmund (18)) in that staphylococcal endocarditis developed regularly only in rabbits receiving preliminary treatments.

Böhmig (19) showed that rabbits injected intracutaneously with small amounts of horse serum reacted subsequently to small intracutaneous inocula of avirulent green streptococci with an inflammation "hyperergic" in character, and succumbed to intravenous inoculations of the same organism in doses which were not fatal for untreated animals.

Daremberg (20) found that the intravenous or subcutaneous injection of 5 cc. of dog serum in guinea pigs infected with human tubercle bacilli occasioned a greatly decreased resistance to later reinfection.

Experiments of Schroeder (10) showed that the production of hemolytic amboceptor, in response to specific antigenic stimulation, was increased by preliminary intravenous injections of pneumococcal autolysates. Similarly, Clark, Zellmer, and Stone (21) found higher agglutination titers for *B. typhosus* after intravenous injections of heat-killed Gram-positive cocci; and Khanolkar (22) encountered a like phenomenon with reference to *B. paratyphosus* after immunizing injections of *B. pyocyaneus* vaccine. Clark and his coworkers noted slight increases in resistance following this heterologous immunization. Analogous findings were reported by Otsuki (23) in that resistance to streptococcal infection was increased by preliminary immunization with *B. coli* and *B. proteus.* It is a commonly known fact that guinea pigs treated with diphtheria toxin plus antitoxin are much more highly sensitized to serum than those treated with normal serum or antitoxin only.

EXPERIMENTAL

The experiments described in this paper demonstrate altered reactivity in rabbits after the disappearance of mild cutaneous streptococcal infections and following intravenous immunization with streptococci. The critical test was the response to intracutaneous injection of small doses of horse serum. Such a test possesses several advantages. Properly controlled, the results are relatively constant; antigenically, horse serum and streptococci have very little in common; in particular, they provoke contrasting types of sensitivity (24, 25). Although small doses have been employed to demonstrate anaphylactic phenomena in guinea pigs, relatively large quantities have been given heretofore to induce the Arthus phenomenon in rabbits (26). We have found, however, that intracutaneous injections of as little as 0.05 cc. stimulate high degrees of cutaneous sensitivity; areas of edema several centimeters in diameter sometimes follow subsequent injections of 0.01 cc. Small doses (0.1 cc.) were used by Böhmig in sensitization; and recently Kahn (27) noted that quantities as small as 0.5 cc. intracutaneously established cutaneous sensitivity. The desirability of employing the smallest possible doses in testing differences in degree of reactivity is obvious.

Variations in response incident to differences in age, sex, and strain of animals employed are described in another communication (28). The influence of these variables was minimized by employing a constant stock of rabbits consisting of a cross between English, lilacs, and Havanas. Four to six animals were used in each group, and groups to be compared with each other were composed of individuals of approximately the same weight. One lot of serum kept without chemical preservative was used throughout.

The scheme of testing is summarized in the following protocol:[1]

Day	Amount of horse serum intracutaneously	Bleeding to obtain serum for precipitins
	cc.	
1	0.1	
3	0.001	
6	0.001	Bleeding
9	0.001	Bleeding
12	0.001	Bleeding
15	0.001	
18	0.001	Bleeding
21	0.001	

A fresh area of skin was selected for each injection. The site of the original injection was observed for 15 days, and those of subsequent injections for 3 days each.

In animals of the character mentioned,[1] the original injection of 0.1 cc. gives rise in 24 hours to a faintly pink papule about 25 mm. in diameter and 1 mm. in height, which fades in 72 hours. In approximately one-half of the animals, however, an edematous lesion reappears at the site of injection about the 9th day and reaches its maximum within 24 hours. This "secondary reaction" is much more intense than the primary reaction and occupies a volume five to ten times as great. It fades completely within a week. The response to the 0.001 cc. injections repeated at 3 day intervals varies. Those made before the 9th day usually provoke no detectable response. Beginning about the 9th day, however, there occur increasingly severe reactions which reach a maximum in 24 hours and fade within a few days. In highly sensitive animals, moreover, a delayed reaction is observed following that injection of 0.001 cc. which was made 48 to 72 hours before the development of the secondary reaction above described. Under such circumstances, for instance, the site of the 0.001 cc. injection made on the 6th day remains negative until the 9th day, when a lesion develops concomitantly with the secondary reaction at the site of the original 0.1 cc. injection. As sensitivity to horse serum increases, lesions resulting from the 0.001 cc. injection develop more rapidly and may reach their maximum within 6 hours. Blood serum precipitins are usually demonstrable 24 to 48 hours after cutaneous sensitivity develops.[2] During the course of such injections immature animals gained weight steadily.

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[1] The intervals indicated here are those suitable for the demonstration of differences in males 2,000 gm. in weight.

[2] Precipitin titers were determined by mixing 0.4 cc. of each saline dilution of horse serum with 0.2 cc. of the rabbit serum to be titrated in small precipitin tubes. The following dilutions of horse serum were employed: 1:100, 1:1,000, 1:5,000,

An increased degree of reactivity to horse serum is made evident in several ways: the primary response is greater; the secondary reaction occurs earlier, is found in a higher proportion of animals, and is more intense; reactions to injections of 0.001 cc. occur sooner and are larger; and "delayed reactions" are occasionally observed. The time of appearance of precipitins in the blood serum is advanced. With decreased reactivity these reactions are delayed or absent.

After sensitivity is established according to the foregoing criteria, the injection of 0.1 or 0.01 cc. of horse serum produces areas of redness and edema sometimes covering one entire side of the trunk and occasionally accompanied by hemorrhage and necrosis.

Altered reactivity was induced by inoculating rabbits in various ways with living cultures of an indifferent streptococcus, which is relatively avirulent for these animals, and also by intravenous immunization with hemolytic streptococci. The various forms of altered response with reference to the infecting microorganism have been described elsewhere (29).

Experiment 1.—In Group B, consisting of five females and one male, a state of bacterial hypersensitivity was induced within a period of 22 days by three daily intracutaneous inoculations in 0.1 cc. volume of from 10^{-2} to 10^{-3} cc. of a culture (Strain Q 155) of indifferent streptococcus diluted in Ringer's solution. At the end of this interval, inocula of 10^{-5} cc., which provoked no lesions at the beginning of the experiment, gave rise to distinct papules, and reactions to larger doses were greatly increased. The blood sera agglutinated the infecting organism in dilution of from 1:1,280 to 1:5,120. The animals had, in effect, been subjected to mild cutaneous streptococcal infections for 3 weeks. They gained weight steadily and at the time of the first horse serum injections[3] showed no evidences of persisting inflammation except small cicatrizing papules in two instances. Group A, comprising six females, received no preliminary treatment. The rabbits all weighed from 1,000 to 1,300 gm. each.

The responses to a test of reactivity to horse serum in these two groups are demonstrated in Chart 1. In the controls (Group A) there were no secondary reactions, precipitins appeared in low titer, and in only half of the animals by the 12th day. The 0.001 cc. test doses first provoked small skin lesions on the 12th day.

1:10,000, 1:50,000, 1:100,000. The tubes were examined after remaining 2 hours at 37°C. in a water bath, and again after 6 to 8 hours in the refrigerator. The slightest definite precipitate not present in a saline control tube was designated as +; heaviest precipitate (rarely encountered) ++++.

[3] Bacterial inoculations were made into the skin over the hind legs and areas over the trunk were used for the horse serum injections.

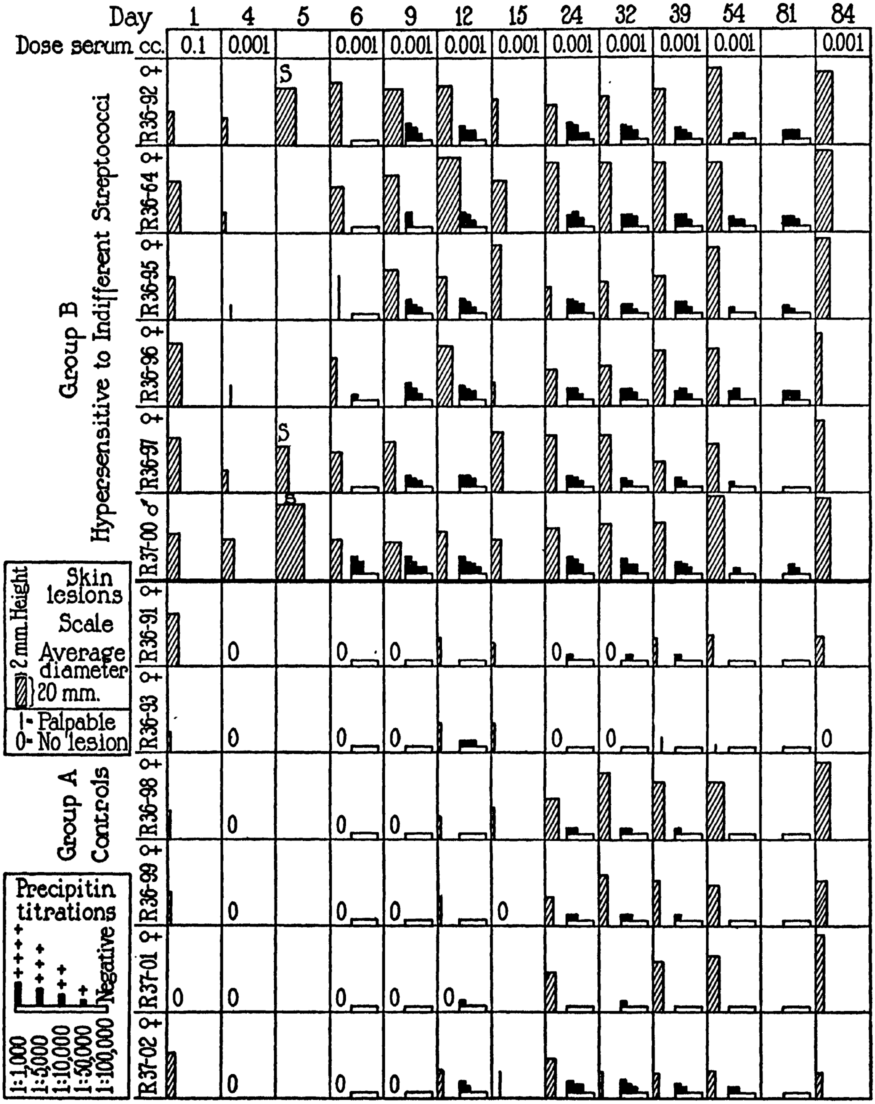

CHART 1. Cutaneous lesions and precipitin titers in control and hypersensitive rabbits following the intracutaneous injection of horse serum. S = secondary reaction.

In contrast, Group B, previously sensitized intracutaneously with streptococci, showed larger original lesions; half of the animals developed secondary reactions on the 4th day; precipitins appeared first on the 6th day and were present in good titer in all by the 9th day. Reactivity to the 0.001 cc. test doses, apparent on the 4th day, became quite marked.

The animals were observed for 3 months; and the following observations concerning the development of skin sensitivity and occurrence of precipitins in the blood serum appear noteworthy.

1. Skin hypersensitivity developed before serum precipitins were demonstrable, especially in the sensitive group (B).
2. As precipitin titers diminished skin sensitivity often persisted or increased in intensity.
3. In individual animals there was no correlation between the degree of skin sensitivity and the height of the precipitin titer.

Experiment 2.—Group B received *intravenous* inocula of Streptococcus Q 155 in the same doses and intervals as those given intracutaneously in the first experiment (total 0.32433 cc. of culture). Meanwhile, the animals steadily gained weight. Their reactivity to horse serum was tested 5 days after the last intravenous inoculation. Cutaneous reactivity to the streptococci and agglutinin titers were not determined; but it was previously demonstrated (30) that in rabbits so inoculated the agglutinin titers are slightly higher than in those receiving comparable small doses intracutaneously, and that although skin reactivity to inoculation with homologous streptococci is very slightly increased, the lesions are of the type encountered in "immunized" animals.

Group C received large immunizing intravenous injections of living cocci. Rabbits so treated develop serum agglutinins in high titer, and upon intracutaneous inoculation with the homologous microorganism show small indurated lesions (29). In this group some animals lost slightly in weight during immunization, but thereafter gained. There was no obvious focal infection, no arthritis, and at autopsy no scars or collections of pus were found; hence it is improbable that any active inflammation was present at the time reactivity to horse serum was tested, *viz.* 5 days after the last intravenous inoculation. At this time a previously untreated control group (A) was introduced. Reactivity to horse serum was tested in the manner used in Experiment 1 with the exception that on the 1st day in addition to the sensitizing dose of 0.1 cc. a test dose of 0.001 cc. was also given. The rabbits weighed 1,500 to 2,000 gm. each and the groups were balanced with regard to weight and sex. The results are indicated in Chart 2.

Control Group A.—There was no reaction to the 0.001 cc. injection on the 1st day. Three of the six rabbits developed secondary reactions, all exhibited a degree of skin sensitivity to the small test doses on the 10th day, and the sera of three of the six showed distinct precipitins on the 16th day.

Group B.—One rabbit reacted slightly to 0.001 cc. of horse serum on the 1st day; three of the five developed secondary reactions, one showed a delayed reaction

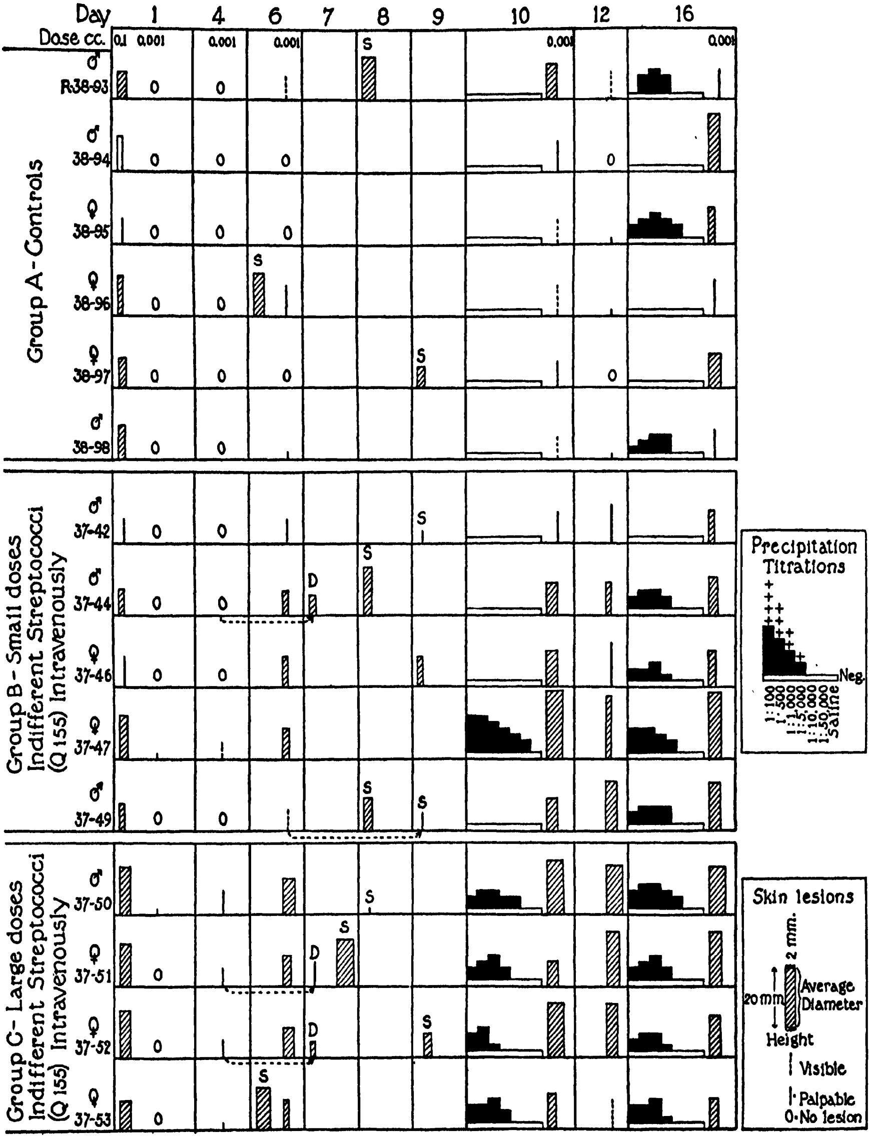

CHART 2. Cutaneous lesions and precipitin titers in rabbits (A) receiving no preliminary treatment, (B) following small doses of indifferent streptococci intravenously, and (C) after large doses of indifferent streptococci intravenously. S = secondary reaction. D = delayed reaction.

to the 0.001 cc. injection on the 4th day, and all gave some inflammatory response to the small test doses on the 6th day. In four of five, precipitins were present on the 16th day, and in one animal precipitins were present in high titer on the 10th day. This group, therefore, showed a very irregular but definite increase in reactivity, although by no means of the grade exhibited in the first experiment by rabbits previously sensitized with comparable doses of the same streptococci intracutaneously.

Group C.—Relatively large lesions resulted from the injections of 0.1 cc. on the 1st day and one animal also responded to 0.001 cc. on this day. Three of the four rabbits developed secondary reactions; all responded to the small test doses on the 4th day (two had delayed reactions to this injection), and all showed distinct precipitins by the 10th day. The increase in reactivity in this group approached but did not equal that shown by the individuals of Group B in Experiment 1 which were subjected to mild cutaneous streptococcal infections for a similar period of time, but which received by comparison only minute doses of the bacteria. This experiment demonstrates the same general phenomena observed in Experiment 1. In addition, delayed reactions to the small test doses were noted frequently, and in some animals showing increased reactivity there was response even to the injection of a 0.001 cc. of horse serum on the 1st day.

Experiment 3.—Here the first two experiments were in principle repeated. One group of rabbits (A) received no preliminary treatment. Group B was sensitized in a manner similar to that used in the first experiment and with cultures of the same strain. Each animal received daily intracutaneous inoculations of from 0.01 to 0.05 cc. for a period of 21 days. The total quantity of culture injected was 0.44011 cc. A third group (C) was given large intravenous immunizing doses of hemolytic streptococci (Strain London M A) which was virulent for rabbits. The first injections were of heat-killed vaccine; later, living cultures were used. Eight weekly series of injections were administered and the individual doses were increased from 1 cc. to 3 cc. during the interval. Group B was tested with horse serum 8 days after the last intracutaneous bacterial inoculation and Group C 2 weeks after the final intravenous immunization. At this time the animals weighed from 1,500 to 2,000 gm.; all were gaining weight and there was no evidence of persisting inflammation in any of them. At autopsy no scars or collections of pus were found to indicate that a chronic infection had been induced.

The results are briefly presented in Table I. The sensitive and immune animals both showed somewhat larger lesions than the controls at the site of the original 0.1 cc. injection. On the 1st day none of the controls reacted to 0.001 cc., while all of the intracutaneously sensitized rabbits and several of the intravenously immunized group showed definite lesions. This order of relative degree of reactivity of the groups, sensitive, immune, control, was also evident in the occurrence of secondary reactions, in the appearance of delayed reactions to 0.001 cc. test doses, in the development of cutaneous sensitivity to repeated test doses, and in the precipitin titers on the 9th day.

TABLE I

The Responses of Control, Hypersensitive, and Immune Rabbits to Small Doses of Horse Serum Intracutaneously

Groups tested with horse serum intracutaneously	Rabbit No.	Cutaneous lesions							Precipitin reaction
		Day 1		Secondary reaction on day	Day 4 — Dose 0.001 cc.		Day 6*	Day 9	Day 9
		Dose 0.1 cc.	Dose 0.001 cc.		Primary reaction	Delayed reaction on Day 6	Dose 0.001 cc.	Dose 0.001 cc.	1:1,000 dilution
Group A	R37-93	19 x 1+	0	8	0	0	15 x v.	24 x 1+	−
	R37-94	25 x 1	0	0	0	0	0	21 x 0.5	−
Controls	R37-95	17 x p.	0	0	0	0	0	15 x v.	−
	R37-96	26 x 1.5−	0	6	0	0	22 x p.	20 x v.	−
	R37-97	20 x 1+	0	9	0	0	0	18 x 0.5+	−
	R37-98	22 x 1−	0	7	0	0	v. & p.	18 x v.	+
Group B	R37-81	24 x 1.5	14 x p.	6	10 x p.	16 x 1	23 x 2	24 x 0.5	+±
Hypersensitive to	R37-82	26 x 1	15 x 1	6	v. & p.	0	26 x 1.5	32 x 3	++
indifferent strep-	R37-84	24 x 1.5	15 x p.	7	v. & p.	0	26 x 2−	31 x 2	++
tococcus	R37-86	27 x 1.5	v. & p.	6	v. & p.	15 x p.	26 x 1.5−	50 x 3.5+	+
	R37-87	30 x 2	10 x p.	7	10 x p.	14 x 1+	19 x 1.5	38 x 3	++
	R37-90	21 x 1+	8 x p.	6	14 x p.	19 x 1.5	30 x 2+	20 x p.	+++
Group C	R37-18	16 x 3	16 x v.	6	8 x p.	0	10 x p.	18 x 1+	+±
Immune to hemo-	R37-19	25 x 1.5	13 x p.	7	0	10 x p.	17 x 1	37 x 2	+±
lytic streptococcus	R37-20	23 x 1+	0	6	v. & p.	−	15 x 1−	56 x 1.5+	++
	R37-23	24 x 2	0	6	0	13 x p.	18 x 2−	54 x 2	++
	R37-24	51 x 3.5	17 x 1+	0	0	0	23 x 1.5	46 x 5	+±

Doses of horse serum intracutaneously (0.1 cc. and 0.001 cc. in 0.1 cc. volume diluted in physiological saline) as indicated. Size of cutaneous lesions: average diameter x estimated height in millimeters.

v. = visible only. p. = just palpable. v. & p. = just visible and palpable.

* Precipitin reactions on 6th day were all negative.

DISCUSSION

The foregoing experiments indicate that antecedent low grade streptococcal skin infections, as well as intravenous immunization with streptococci, alter the immunological responses of rabbits to horse serum in the direction of accelerated reactivity. They do not, however, clarify the mechanism responsible for the establishment of such an altered state. Lewis and Loomis (8) have proposed the term "allergic irritability" for a similar condition developing *during* infection and have defined it as the "capacity of the animal to react to antigenic substances" and "as a general characteristic of the animal on the basis of which it reacts to stimuli of the antigenic class whether they be helpful, injurious or indifferent to bodily health." These authors, as well as Dienes, Siegmund, and others, have suggested that the stimulation of the reticulo-endothelial system during infection may be responsible for the phenomena observed. They describe an experiment in which trypan blue injections in rabbits had an effect similar to infection in accelerating the production of hemolytic amboceptor.

The probability of the correctness of such an explanation has increased with the further development of knowledge concerning the function of the reticulo-endothelial system. The voluminous literature dealing with so called anamnestic reactions is reviewed by Topley (31) and by Weichardt (32). Extensive experiments, particularly those of Walbum (33) and of Weichardt and their collaborators, have indicated that the intravenous injection of metallic salts, colloids, or of protein solutions, may, in certain phases of the immunization process with various microorganisms, increase the titer of circulating antibodies. The significance of such observations indicating that measures affecting the state of reactivity of the reticulo-endothelial system are of decisive influence in altering the immunological response, is discussed in a recent review by Jungeblut (34). The stimulation of this cellular system, as in the demonstration of "anamnestic reactions" results in increased immunological reactivity. Depression has been found to retard the development of skin sensitivity (35) and to prevent fatal tuberculin shock in guinea pigs (36, 37). Numerous studies involving extirpation of the spleen and blockade with various substances indicate that an intact reticulo-endothelial system is essential for normal antibody formation.

Most of the experiments cited have dealt almost exclusively with effects of intravenous treatment or inoculation in altering the functions of the reticulo-endothelial system. The stimulating influence of streptococcal sepsis on this system in the rabbit has been particularly noted (38). That mild local cutaneous infections should have a similar effect might be expected, but our experiments indicate that they exert a considerably more marked influence than that aroused by intravenous stimulation. Tuft (39), recently, in calling attention to the importance of the skin as an immunological organ, suggests that this may be due to the local stimulation of cells of the reticulo-endothelial system which are especially abundant there. The effect of *localized* inflammation in causing a generalized stimulation of reticulo-endothelial structures has been noted in histologic studies of the liver (40) and of the bone marrow (41), and in physiological experiments (42).

There is abundant evidence that the development of anaphylactic phenomena is distinctly modified by the state of reactivity of the reticulo-endothelial system. For example, Klinge (43) in studying the pathogenesis of the Arthus phenomenon in rabbits sensitized with horse serum, found that the local development of hyperergic inflammation was prevented when the cells of the reticulo-endothelial system, characteristically involved in the reaction, had become impregnated with trypan blue.

It seems justifiable, therefore, to conclude that the accelerated response to intracutaneous injections of horse serum observed in the experiments reported here was conditioned by a state of hyperreactivity of the reticulo-endothelial system which had been induced by the antecedent streptococcal infections and injections. It may be that a similar conditioning of reactivity explains, in part, certain phenomena observed by Jones and Mote (44) in patients suffering from rheumatic fever. These authors noted that such patients became hypersensitive to rabbit serum after receiving repeated intradermal injections of very small amounts of this foreign protein. It seems highly probable that the rheumatic state accelerated and altered the mode of reactivity of these children to the test substance.

An analogy to the phenomena demonstrated in the experiments described is perhaps found in the broadening of the zone of hypersensitivity known from experience to occur in patients. Sensitizations caused by one substance tend to lose their monovalence and become polyvalent, with a demonstrable sensitivity of the skin, for instance, embracing many substances which may or may not be of clinical significance.

SUMMARY

1. The cutaneous responses of rabbits to small doses of horse serum intracutaneously is described. After an original injection of 0.1 cc. a secondary reaction often occurs about the 9th day, and tests at 3 day intervals with 0.001 cc. quantities indicate that general skin hypersensitivity is established at this time. Circulating precipitins for horse serum appear later. As the degree of sensitivity increases, lesions resulting from test doses reach a maximum development more quickly.
2. The reactivity of rabbits to horse serum is greatly increased by antecedent sensitization of the animals with repeated small intracutaneous inoculation of indifferent streptococci or immunization with large intravenous injections of either indifferent or hemolytic streptococci. Doses of indifferent streptococci precisely comparable to those producing greatly enhanced reactivity when given intracutaneously, increase reactivity to horse serum irregularly and to slight degree when injected intravenously.
3. Increases in reactivity (allergic irritability) are made evident by the occurrence of larger skin lesions at the site of the primary horse serum injections, the earlier development of more distinct secondary reactions in a higher percentage of animals, and by the more rapid appearance of skin sensitivity to the small test doses which may be first evidenced by delayed reactions at these injection sites. Accompanying these evidences of increased reactivity, there is an early appearance of blood serum precipitins in high titer.
4. There was no evidence of residual inflammation in any of the animals at the time reactivity to horse serum was tested. No attempt was made to determine the duration of this altered reactive capacity. It has been encountered in tests begun from 8 to 14 days after the last preliminary inoculation.
5. It is suggested that the increased reaction described is brought about by a persisting alteration in the functional activity of the reticulo-endothelial system.

BIBLIOGRAPHY

1. Levinson, S. A., and Petersen, W. F., *Am. Rev. Tuberc.*, 1927, **15**, 681.
2. Kauffmann, F., *Klin. Woch.*, 1928, **7**, 1309.
3. Dienes, L., *J. Immunol.*, 1927, **14**, 61.
4. Lewis, P. A., and Loomis, D., *J. Exp. Med.*, 1924, **40**, 503.
5. Selter, H., *Schrift. Königsber. gelehrten Ges.*, 1926, **7**, 137.
6. Hektoen, L., and Corper, H., *J. Infect. Dis.*, 1925, **37**, 82.
7. Dold, H., and Gross, H., *Centr. Bakt., 1. Abt., Orig.*, 1927, **104**, 343.
8. Lewis, P. A., and Loomis, D., *J. Exp. Med.*, 1926, **43**, 263.
9. Blumenberg, W., *Z. ges. exp. Med.*, 1926, **49**, 500.
10. Schroeder, M. L., *J. Med. Research*, 1924, **44**, 507.
11. Wessely, K., *Münch. med. Woch.*, 1911, **58**, 1713.
12. Löwenstein, A., *Klin. Monatsbl. Augenheilk.*, 1927, **78**, suppl., 73.
13. Igersheimer, J., *Zentr. ges. Ophth.*, 1931, **26**, 1; *Arch. Ophth.*, Leipsic, 1913, **85**, 316.
14. Gildmeister, E., and Schlossberger, H., *Centr. Bakt., 1. Abt., Orig.*, 1933–34, **130**, 518.
15. vom Hofe, K., *Klin. Monatsbl. Augenheilk.*, 1932, **89**, 28.
16. vom Hofe, K., and Krantz, W., *Arch. Augenheilk.*, 1932, **105**, 721.
17. Semsroth, K., and Koch, R., *Arch. Path.*, 1929, **8**, 921.
18. Siegmund, H., *Virchows Arch. path. Anat.*, 1933, **290**, 3.
19. Böhmig, R., *Klin. Woch.*, 1933, **12**, 258.
20. Daremberg, quoted by Zironi, A., *Ergebn. Hyg., Bakt., Immunitätsforsch., u. exp. Therap.*, 1933, **14**, 561.
21. Clark, P. F., Zellmer, C. E., and Stone, H. W., *J. Infect. Dis.*, 1922, **31**, 215.
22. Khanolkar, V. R., *J. Path. and Bact.*, 1924, **27**, 181.
23. Otsuki, S., *Z. Immunitätsforsch.*, 1932, **74**, 249.
24. Derick, C. L., and Swift, H. F., *J. Exp. Med.*, 1929, **49**, 615.
25. Dienes, L., and Mallory, T. B., *Am. J. Path.*, 1932, **8**, 689.
26. Zinsser, H., Infection and resistance, New York, The Macmillan Co., 3rd edition, 1923.
27. Kahn, R. L., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 603.
28. Schultz, M. P., *J. Exp. Med.*, 1934, **60**, 339.
29. Swift, H. F., and Derick, C. L., *J. Exp. Med.*, 1929, **49**, 883.
30. Schultz, M. P., and Swift, H. F., *J. Exp. Med.*, 1932, **55**, 591.
31. Topley, W. W. C., and Wilson, G. S., The principles of bacteriology and immunity, London, Edward Arnold, 1929, 2 vols.
32. Weichardt, W., *Ergebn. Hyg., Bakt., Immunitätsforsch., u. exp. Therap.*, 1922, **5**, 275.
33. Walbum, L. E., *Z. Immunitätsforsch.*, 1926, **47**, 213; 1927, **49**, 538.
34. Jungeblut, C. W., *Ergebn. Hyg., Bakt., Immunitätsforsch., u. exp. Therap.*, 1930, **11**, 1.
35. Freund, J., *J. Immunol.*, 1926, **11**, 382.
36. Jungeblut, C. W., *J. Exp. Med.*, 1928, **47**, 261.

37. Walbum, L. E., *Compt. rend. Soc. biol.*, 1926, **94**, 1106.
38. Voicu, J., Vitalyos, A., and Boer, L., *Virchows Arch. path. Anat.*, 1933, **288**, 455.
39. Tuft, L., *J. Immunol.*, 1931, **21**, 85.
40. Hess, W., *Frankf. Z. Path.*, 1931, **42**, 89.
41. Konstantinov, V., *Arch. sc. biol.*, 1930, **30**, 659.
42. Eichbaum, F., and Scheufler, C., *Frankf. Z. Path.*, 1932–33, **44**, 10.
43. Klinge, F., *Krankheitsforschung*, 1927–28, **5**, 308, 458.
44. Jones, T. D., and Mote, J. R., *New England J. Med.*, 1934, **210**, 120.

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THE INFLUENCE OF SEXUAL MATURITY UPON THE REACTIVITY OF RABBITS TO HORSE SERUM

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Many observations indicate that immunological reactivity is conditioned to some extent by the degree of sexual maturity. In certain tropical and arctic communities where scarlet fever and diphtheria occur very rarely, negativity to both Schick and Dick tests has been found to develop at puberty. Certain dermatomycoses occur exclusively in childhood and heal spontaneously at puberty (1). The development of antibodies against antigen with which contact is not ordinarily established has been found to be similarly conditioned. Conflicting interpretations of such observations are discussed by Manwaring (2), Jungeblut (3), and Herrman (4).

The incidence, severity, and character of the manifestations of rheumatic fever are apparently influenced by factors associated with age and sex. Most observers agree that the disease is chiefly prepubertal in origin, some studies (5, 6) indicating that 98 per cent of first attacks occur before the ages of 12 or 15. These investigations also show that the tendency to relapse is greater in the cases earliest in onset and that in childhood, especially in girls, visceral disease (including carditis) occurs frequently with little or no concomitant arthritis. Chorea develops much more commonly in females but is rarely encountered after puberty except as a complication of pregnancy (7). Wilson, Lingg, and Croxford (6), among others, have stressed the fact that about the 12th year the tendency to rheumatic infection begins to diminish. A recent study by von Eickstedt (8) concerning the influence of puberty showed that among 24 girls there were 16 recurrences of the disease in the 4 year period before puberty and none in the following 4 years; among 14 boys the figures were 11 before and 2 afterwards.

The statement is frequently made that the incidence and severity of rheumatic fever is greater in females than in males of all age groups. Although several general statistical compilations indicate that this is the case, a critical review of the more reliable sources does not substantiate this unqualified conclusion. While most investigations demonstrate that all of the rheumatic manifestations are more frequent and severe in girls than in boys under 12 years of age, those dealing with children under 15 or including all age groups are conflicting in their conclusions regarding the influence of sex factors. The relatively greater drop in the incidence of the manifestations of rheumatic fever in females at puberty may be attributed either to a greater influence of the attainment of sexual maturity in that sex or to the fact that while boys and girls are rather equally subjected to environmental factors, different influences are active in the two sexes in adult life; for although women are subjected to the varying influences of the menstrual cycle, men are somewhat more exposed to the stress and strain incident to hard labor and inclement weather.

In the investigations dealing with the influence of age on the formation of natural antibodies or on the response to active immunization (most recently summarized[1] by Freund (9) and Kligler and Olitzki (10)), infants have been compared with adults, and both the intervening age periods and sex factors have, with few exceptions, been neglected. The purpose of the present experiments was to study the influence of the phase of maturation in the two sexes.

Friedberger (11), observing rabbits at intervals from birth to maturity, found that natural anti-sheep erythrocyte hemolysin (amboceptor) which was present at birth, disappeared within 8 days and reappeared thereafter in gradually increasing titer until about the 88th day, with a subsequent slight fall. The time of maximal concentration coincided approximately with that at which sexual maturity is usually established.

Kligler and Olitzki, in titrating antibodies following subcutaneous injections of typhoid vaccine in guinea pigs weighing from 245 to 770 gm., found that the highest titers were attained in animals of 475 to 520 gm. in weight. Agglutinins and complement increased in strength among younger individuals with increasing age: bacteriolysins were first detected in animals weighing 300 gm.; and both agglutinins and bacteriolysins decreased slightly in those over 520 gm. Sexual maturity in guinea pigs, as indicated by the onset of estrus cycles, is attained at the earliest with a weight of 300 gm. and is rarely absent in animals weighing over 500 gm.[2] The maximal increases in antibodies described took place in this interval. In rats ranging from 26 to 114 gm. in weight, similarly immunized, the results were analogous. The highest titers were attained in those weighing between 64 and 83 gm. Sexual maturity in rats (12) as indicated by the onset of an abrupt gain in weight of the ovaries begins when a weight of about 60 gm. has been attained. The relatively greater increases in antibodies in this species began at that point.

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[1] Later observations showing differences in immunological reactivity between young and mature rabbits were reported in two abstracts by Baumgartner and by Kahn and McDermott, at the 1934 meeting of the American Association of Immunologists. See *J. Immunol.*, 1934, **26**, 327.

Burky (13) observed that rabbits less than 4 months old were insusceptible to the toxic action of a very poisonous broth filtrate of staphylococci, while the majority of rabbits above this age reacted characteristically to intracutaneous injections and succumbed after receiving this toxin intravenously.

The observations mentioned indicate that immunological reactivity is greater in adults than in infants, and that the major increase takes place concomitantly with the phase of sexual maturation. The maximal degree of immunological reactivity, however, seems to be reached just at the point at which physiological sexual maturity is attained, with a subsequent slight decline during continued adult life. It is to be borne in mind that in the experiments quoted no distinction was made between the sexes.

EXPERIMENTAL

In the experiments described here, rabbits of both sexes and of varying ages are compared in their responses to small doses of horse serum given intracutaneously. The method employed and the character of the reactions studied are described in detail elsewhere (14). Briefly, an original injection of 0.1 cc. is followed at intervals of 3 days by repeated doses of 0.001 cc. The establishment of hypersensitivity to horse serum is indicated by the occurrence of a secondary reaction at the site of the original injection, by the development of lesions following the repeated test doses, and by the appearance of blood serum precipitins. Increased reactivity of the individual is manifest by an acceleration and intensification in the development of the signs of hypersensitivity. The rabbits compared were for the most part of a hybrid English-lilac-Havana stock, bred and raised under identical conditions at The Rockefeller Institute. One group only consisted in adult New Zealand Reds secured from a dealer. Although the age of most of the animals was known, it has been found more convenient to express the degree of development by weight rather than by age.

Experiment 1.—Seventeen male rabbits ranging in weight from 980 to 3,050 gm. were treated with horse serum in the manner outlined. The results are indicated in Table I. In the 13 individuals weighing less than 1,700 gm. no secondary reactions occurred, only small lesions developed in 4 animals following test doses on the 6th and 9th days; and blood serum precipitins were distinctly present in only one instance on the 15th day. In contrast, those weighing 1,700 gm. or more all developed secondary reactions, most of them responded to small test doses given on the 6th day, while all showed quite intense lesions to tests on the 9th day; and each individual showed precipitins in good titer by the 15th day. An acceleration and an intensification in the development of sensitivity is quite evident in the males weighing more than 1,700 gm.

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[2] Personal communication from Professor C. R. Stockard.

TABLE I

Responses of Male Rabbits to Small Doses of Horse Serum Intracutaneously

Rabbit No.	Weight	Cutaneous lesions				Precipitin reactions		
		Secondary reaction on day	Dose*			1:1,000 dilution		
			0.001 cc.	0.001 cc.	0.001 cc.			
			Day 3	Day 6	Day 9	Day 6	Day 9	Day 15
	gm.							
R37-87	950	0	0	0	0	−	−	±
R37-86	980	0	0	0	0	−	−	+
R37-85	1,000	0	0	0	20 x v.	−	−	±
R37-83	1,075	0	0	0	0	−	−	±
R37-84	1,080	0	0	0	0	−	−	−
R37-79	1,135	0	0	0	0	−	±	±
R37-82	1,170	0	0	0	0	−	−	±
R37-77	1,170	0	0	0	0	−	−	±
R37-80	1,180	0	0	0	0	−	−	±
R37-75	1,375	0	0	0	7 x p.	−	−	±
R37-03	1,475	0	0	0	0	−	−	±
R36-79	1,480	0	0	12 x p.	0	−	−	±
R37-94	1,550	0	0	0	21 x 0.5	−	−	−
R36-93	1,700	8	0	15 x v.	24 x 1+	−	−	++
R36-98	1,850	7	0	0	15 x v.	−	+	+±
R37-33	2,860	9	0	13 x 0.5	22 x 2	−	−	++±
R37-55	3,050	9	0	20 x 1.5	22 x 1.5+	−	−	++±

Size of cutaneous lesions: average diameter x estimated height in millimeters. v = visible only; p = just palpable.

Animals indicated below the double line weighed 1,700 gm. or more.

* Doses of horse serum intracutaneously: 0.1 cc. on 1st day, and 0.001 cc. as indicated.

Experiment 2.—Thirteen female rabbits weighing from 990 to 4,080 gm. were tested in a similar fashion. As indicated in Table II, cutaneous lesions developed and precipitins appeared irregularly within the group. There was no indication that the degree of reactivity increased with advancing age.

Experiment 3.—In further studies males were compared with females of approximately the same age. The results, including animals used in Experiments 1 and 2, are shown in Table III. In Group A males and females weighing less than 1,700 gm. each are compared. The cutaneous lesions following the test dose on the 9th day were of about equal intensity, and the precipitin titrations on the 15th day were similar. As may be noted also in Experiments 1 and 2, the degree of reactivity was about the same in both sexes before a weight of 1,700 gm. was reached.

TABLE II

Responses of Female Rabbits to Small Doses of Horse Serum Intracutaneously

Rabbit No.	Weight	Cutaneous lesions				Precipitin reactions			
		Secondary reaction on day	Dose			1:1,000 dilution			
			0.001 cc.	0.001 cc.	0.001 cc.				
			Day 3	Day 6	Day 9	Day 6	Day 9	Day 12	Day 15
	gm.								
R36-90	990	0	0	0	17 x 0.5	–	–		±
R36-78	1,500	0	0	0	0	–	–		–
R36-91	1,500	0	0	0	0	–	–	–	
R37-01	1,530	0	0	0	0	–	–	+	
R36-97	1,550	9?	0	0	18 x 0.5+	–	–		–
R36-93	1,600	0	0	0	0	–	–	+	
R36-99	1,850	0	0	0	0	–	–	+	
R37-02	1,880	0	0	0	0	–	–	+±	
R37-98	1,900	0	0	0	0	–	–	–	
R37-95	1,950	0	0	0	15 x v.	–	–		–
R37-96	2,000	6	0	22 x p.	20 x v.	–	–		++
R37-30	3,470	0	0	0	0	–	–		–
R37-31	4,080	0	0	0	0	–	–		–

In Group B males and females weighing over 1,700 gm. are compared. The older females developed sensitivity in a manner comparable to the younger individuals of both sexes. In contrast, the males over 1,700 gm. in weight showed secondary reactions, and distinct lesions to small test doses on the 6th and 9th days, while precipitins were uniformly present in good titer by the 15th day. Group C was similar to Group B in respect of maturity, but received an original sensitizing dose of 0.2 cc. instead of 0.1 cc. The same differences in reactivity, however, were manifest: In individuals weighing more than 1,700 gm. sensitivity developed more quickly and more intensely in males.

The results of these experiments indicate that male and female rabbits respond to horse serum in about the same manner before they have attained a weight of 1,700 gm.; that is, to about the age of 13 weeks. With advancing age beyond this point only males show an increasing reactivity. The situation may be represented schematically as shown in Chart 1.

TABLE III

Responses of Male and Female Rabbits of Different Age Groups to Small Doses of Horse Serum Intracutaneously

	Rabbit No.	Weight	Sex	Secondary reaction on day	Cutaneous lesions			Precipitin reactions		
					Dose			1:1,000 dilution		
					0.001 cc.	0.001 cc.	0.001 cc.			
					Day 3	Day 6	Day 9	Day 6	Day 9	Day 15
		gm.								
Group A Under 1,700 gm. in weight	R37-90	990	Female	0	0	0	17 x 0.5	–	–	±
	R37-87	1,500	"	0	0	0	0	–	–	–
	R37-97	1,550	"	9	0	0	18 x 0.5+	–	–	–
	R37-86	980	Male	0	0	0	0	–	–	+
	R37-82	1,170	"	0	0	0	0	–	–	±
	R37-94	1,550	"	0	0	0	21 x 0.5	–	–	–
Group B Over 1,700 gm. in weight	R37-95	1,950	Female	0	0	0	15 x v.	–	–	–
	R37-96	2,000	"	6	0	22 x p.	20 x v.	–	–	++
	R37-30	3,470	"	0	0	0	0	–	–	–
	R37-31	4,080	"	0	0	0	0	–	–	–
	R37-93	1,700	Male	8	0	15 x v.	24 x 1+	–	–	++
	R37-98	1,850	"	7	0	0	15 x v.	–	+	+±
	R37-35	2,860	"	9	0	20 x 1.5–	22 x 1.5+	–	–	++±
	R37-33	3,050	"	9	0	13 x 0.5	22 x 2	–	–	++±
Group C* Over 1,700 gm. in weight	R37-28	4,085	Female	10	0	0	18 x 0.5	–	–	+
	R37-29	4,190	"	10	0	0	17 x 1	–	–	–
	R37-32	2,830	Male	7	0	20 x 2	32 x 2.5	–	–	++
	R37-34	3,080	"	8	0	16 x 1	22 x 1.5+	–	–	++

* Group C received 0.2 cc. of horse serum on 1st day.

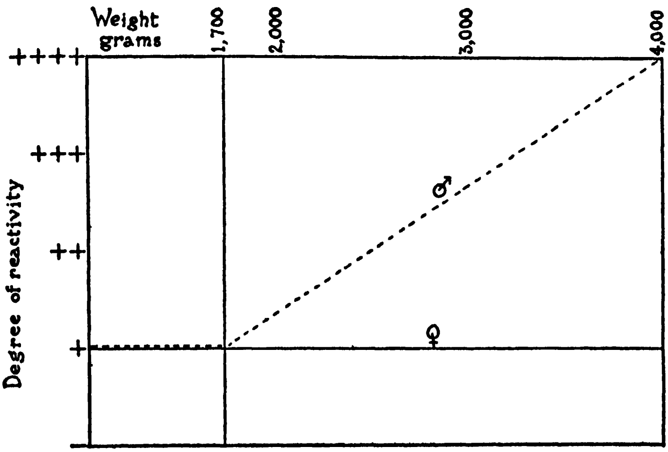

CHART 1

In attempting an analysis of the divergence in character of response with advancing age in the sexes two possible explanations suggest themselves: (1) There are factors peculiar to the male associated with physiological maturation which are responsible for increasing immunological reactivity in that sex. (2) The effect of physiological maturation obtains in both sexes but is not evident in the female because a coincident nullifying influence comes into play. The latter explanation is rendered plausible by the fact that females of the stock employed usually go into heat at the age of 13 weeks, when a weight of 1,700 gm. has been attained, while it is just at this stage of development that the divergence between the sexes in respect of reactivity to horse serum is first manifest. In view of this fact, and because the factor of estrus can be subjected to experimental investigation the following experiment was devised.

Experiment 4.—Twelve adult rabbits (2,300 to 3,000 gm.) were divided into three groups: Group A comprised 4 males which were subjected to simple laparotomy; Group B, 4 females similarly handled; and Group C, 4 females subjected to ovariectomy. 2 weeks after the operations, the animals were all tested with horse serum, and observations were continued for 5 weeks. Because low grade streptococcal infections have been found to increase reactivity to horse serum (13), the wounds were observed closely during life and examined carefully at autopsy to determine whether or not infection had developed. No correlation, however, was found between the amount of inflammation in the wounds and the degree of reaction to horse serum.

The results of the tests with horse serum are shown in Table IV. One-half the males (Group A) had secondary reactions and 3 of them showed cutaneous reactions to test doses on the 9th day. In one animal precipitins were detected on the 12th day, and they were present in good titer in 3 by the 29th day. All the castrated females (Group C) showed secondary reactions and slight cutaneous lesions to test doses on the 9th day. Precipitins appeared in about the same concentration as in Group A. The females subjected to laparotomy only (Group B), in contrast to the animals in the other two groups, had no definite secondary reactions, developed no distinct lesions to the test

TABLE IV

Responses of Male, Female, and Ovariectomized Female Rabbits to Small Doses of Horse Serum Intracutaneously

	Rabbit No.	Secondary reaction on day	Cutaneous lesions			Precipitin reactions			
			Dose			1:1,000 dilution			
			0.001 cc.	0.001 cc.	0.001 cc.				
			Day 9	Day 12	Day 15	Day 12	Day 15	Day 21	Day 29
Group A Laparotomized males	R39-66	8	20 x 0.5*	22 x 1	24 x 2	+	++	++	+++
	R39-67	9	12 x v.	15 x v.	18 x 0.5	−	−	−	+±
	R39-68	0	21 x 0.5	21 x 1	18 x 0.5	−	−	+	++
	R39-70	0	0	18 x v.	18 x p.	−	−	−	−
Group B Laparotomized females	R39-56	10?	v. & p.	22 x 1	32 x 2	−	−	−	−
	R39-59	0	0	0	v. & p.	−	−	−	+
	R39-61	0	0	26 x p.	21 x 0.5	−	±	++	++
	R39-63	0	0	17 x p.	18 x 0.5	−	−	±	−
Group C Ovariectomized females	R39-54	11	10 x p.	15 x 1	20 x p.	−	−	+	+±
	R39-55	9	13 x p.	17 x 0.5	19 x 0.5	−	−	+±	+±
	R39-57	8	10 x p.	18 x 0.5	20 x 0.5	−	−	−	+
	R39-60	6	23 x p.	26 x 0.5	25 x 0.5	+	+	+±	++

* Definitely elevated lesions are underlined.

doses on the 9th day, and showed in only one individual precipitins in concentration comparable to that in the other groups. The findings indicate that adult female rabbits with intact ovaries develop sensitivity to horse serum more slowly and less intensely than do males or castrated females.

At operation the uteri of all the animals in Group B were large and red, indicating that at the start of the experiment all members of this group were in heat. Autopsies were not performed until 2½ months later, but at that time the uteri in Group B were large and red in contrast to the atrophic pale uteri in Group C. There was no proof, however, that such a uniform difference existed in the uteri of the two groups several weeks earlier when differences in reactivity to horse serum were being manifest. It may be noted, also, that in general skin lesions were smaller than in unoperated animals, suggesting that a 2 week interval may be an insufficient period for recuperation from the

TABLE V

Responses of Laparotomized and Ovariectomized Female Rabbits to Small Doses of Horse Serum Intracutaneously

Rabbit No.	Operation	Weight of uterus and vagina	Uterus weight per cent of body weight*	Cutaneous lesions					Precipitin reactions
				Secondary reaction on day	Dose				
					0.001 cc.	0.001 cc.	0.001 cc.	0.001 cc.	1:1,000
					Day 5	Day 6	Day 9	Day 12	Day 12†
		gm.	*per cent*						
R38-78	Laparotomy	11.0	0.48	0	0	0	20 x 1	0	−
R38-77	"	11.25	0.42	0	0	0	15 x 0.5	25 x 0.5−	−
R38-80	Ovariectomy	8.25	0.38	9?	0	0	20 x 1+	35 x 1.5	−
R38-75	Laparotomy	7.5	0.36	9	0	16 x 1−	21 x 1+	24 x v	+
R38-76	"	7.5	0.28	8	16 x 0.5	20 x 1+	33 x 2.5	55 x 2	++
R38-85	Ovariectomy	5.25	0.20	6	22 x 1.5	18 x 1.5	42 x 3	40 x 2.5	++
R38-86	"	4.0	0.16	6	0	20 x 1.5	47 x 3	53 x 3−	+++
R38-81	"	3.5	0.13	7	12 x v.	17 x 1	23 x 1.5	30 x 2	++

* $\frac{\text{Weight of uterus and vagina}}{100 \text{ gm. body weight}}$.

† Precipitin reactions on 9th day were all negative.

effects of operation in experiments of this character. A fifth experiment was, therefore, devised in which these disturbing factors were reduced to a minimum.

Experiment 5.—Eight adult female rabbits were subjected to laparotomy at which time the ovaries were removed from 4 of them. A period of 1 month was allowed for recovery, and autopsies were performed 13 days after the first injection of horse serum. In other particulars, Experiment 4 was repeated. The results are shown in Table V, ordered according to the degree of estrus present in each animal as evidenced by the relative weights of the uteri at autopsy. Those at the top of the list had relatively large uteri and were definitely in heat; those at the bottom with small uteri were not. It is to be noted that in one instance (R 38-80) the object of the ovariectomy was not accomplished, for although at autopsy no residual ovarian tissue could be identified grossly, the general appearance of the uterus indicated that the animal was in heat. In at least one instance (R 38-76) although the ovaries were intact the uterus was not in the estrus stage.

Probably in consequence of the long recuperative interval after operation in this experiment, cutaneous lesions developed in characteristic fashion. The test with horse serum indicated a distinct difference in degree of reactivity between the animals definitely in heat and those in which atrophy of the uterus had taken place, with a gradual transition between the two extremes. The two individuals (R 38-78 and R 38-77) with very large uteri and intact ovaries had no secondary reactions, showed only slight cutaneous lesions to test doses of 0.001 cc. beginning on the 9th day, and did not develop precipitins by the 12th day. The 3 castrated rabbits with very small uteri (R 38-85, R 38-86, and R 38-81) all had early secondary reactions, showed skin reactivity to test doses of 0.001 cc. beginning on the 5th day and becoming quite intense on the 9th and 12th days, and all developed precipitins in good titer by the 12th day.

DISCUSSION

These experiments indicate that in male rabbits of the stock employed reactivity to horse serum injected intracutaneously increases with age after 13 weeks, or, in other words, after a weight of approximately 1,700 gm. is attained. In normal females in estrus this increase does not occur with advancing age. Although castrated females showed increasing reactivity coincident with the absence of estrus, the degree of increase was insufficient to account for the divergence exhibited between adult male and female rabbits. It seems probable, therefore, that there may be some additional factor besides estrus to account for the differences in reactivity in the two sexes after attaining maturity.

Friedberger's work demonstrating that in rabbits there is an increase in normal hemolytic amboceptor from the 8th to the 88th days of life with a subsequent slight fall has been mentioned. An inspection of the protocols of his experiments indicates that although the rise in titer was evident in each of the 20 animals studied, the slight fall after the 88th day was confined to the 15 females, while the concentrations of this antibody continued to rise irregularly in the 5 males. In our experiments, the divergence between males and females occurred after the 13th week (91st day). Friedberger's experiments, therefore, appear to indicate that the concentration of normal hemolytic amboceptor is affected by the same factors which influence reactivity to horse serum.

We have found no other record of analogous immunological observations. The experiments of Barron (15) indicate, however, that reactivity to drugs may be conditioned in a comparable manner by the character of sexual maturity. In administering sodium amytal to rats varying between 30 and 400 gm. in weight, he found that the quantity required to produce deep non-fatal anesthesia became greater with increasing weight in males. Females responded similarly until a weight of 60 gm. was reached, but thereafter they required a relatively smaller quantity. The tolerance of males beyond this period, on the other hand, increased sharply. This sex difference appeared at an age when the ovaries begin to increase rapidly in weight.

SUMMARY

1. Normal rabbits in the course of their development, after infancy, vary in their responses to minute doses of horse serum given intracutaneously.
2. There is increased reactivity in males as they mature, beginning, in the stock employed in these experiments, at about the age of 13 weeks when a weight of 1,700 gm. has been attained.
3. The reactivity of normal females remains at a constant level and the increase demonstrated in males does not take place.
4. The divergence in degree of reactivity between males and females, beginning with the establishment of estrus, indicates that factors associated with sexual maturity in females are responsible for their low reactivity.

5. The suppression of estrus by operative removal of the ovaries results in an increase of reactivity which, however, in the experiments reported here is probably not great enough to account entirely for the difference in reactivity between mature males and females.

BIBLIOGRAPHY

1. Bloch, B., *Schweiz. med. Woch.*, 1933, **14**, 404.
2. Manwaring, W. H., *Science*, 1931, **74**, 324.
3. Jungeblut, C. W., *J. Am. Med. Assn.*, 1932, **99**, 2091.
4. Herrman, C., *Arch. Pediat.*, 1932, **49**, 785.
5. Smith, C. H., and Sutton, L. P., Heart disease in infancy and childhood, New York, Appleton, 1930.
6. Wilson, M. G., Lingg, C., and Croxford, G., *Am. Heart J.*, 1928, **4**, 164.
7. Willson, P., and Preece, A., *Arch. Int. Med.*, 1932, **49**, 671.
8. von Eickstedt, E., *Z. Kinderheilk.*, 1934, **56**, 64.
9. Freund, J., *J. Immunol.*, 1930, **18**, 315.
10. Kligler, I. J., and Olitzki, L., *Z. Hyg. u. Infectionskrankh.*, 1929, **110**, 459.
11. Friedberger, E., and Gajzago, D., *Z. Immunitätsforsch.*, 1930, **67**, 67.
12. Donaldson, H. H., The rat, Memoirs of The Wistar Institute of Anatomy and Biology, No. 6, Philadelphia, 2nd edition, 1924.
13. Burky, E. L., *J. Immunol.*, 1933, **24**, 127.
14. Schultz, M. P., and Swift, H. F., *J. Exp. Med.*, 1934, **60**, 323.
15. Barron, D. H., *Science*, 1933, **77**, 372.

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A COMPARISON OF OCULAR MICROMETRIC AND PROJECTOSCOPIC METHODS OF ESTIMATING GROWTHS IN TISSUE CULTURES

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An important part of investigations dealing with tissue cultures is the estimation of the amount of growth occurring under a given set of conditions; and for this purpose the procedure described by Ebeling (1) has proven the most satisfactory. Those employing this method recognize that it does not give an accurate picture of the total number of cells that have grown in the culture, because the areas drawn represent extension in only two dimensions, and fail to consider the third as represented by the thickness of the growth. The critics of this procedure, nevertheless, have offered no practical substitute; and many investigations continue to be recorded in which no quantitative criteria are applied. The microscopic appearance of the cells is obviously important in helping one to form a final judgment, and should not be neglected. If, therefore, the data for both quantitative and qualitative criteria could be gathered from a single manipulation, certain distinct advantages would follow. The purpose of this communication is to describe a simple method whereby fairly satisfactory quantitative data can be easily acquired.

The method of Ebeling necessitates considerable apparatus. For the drawing of the silhouette some form of projectoscope is required, and if the source of light is very hot the culture must be protected by a heat-absorbing chamber. Unless the apparatus is in a warm room the culture flasks or slides are subjected to cooling with a consequent retarding of growth. The accuracy of the results depends, naturally, upon the experience of the observer in outlining the silhouettes and in planimetry, and also upon an understanding of the relationships of these two maneuvers one to the other. In fact, the limitations of tissue culture technique make too minute measurements unnecessary. A planimeter is, however, needed for the determination of the area occupied by the drawn silhouette. As eight or more growths are generally used in each portion of an experiment, the time required for making tracings and measurements is no small item; and this must be followed by calculation of increments of growth and of comparative indices. Usually the increment represents the average of calculations from a number of growths, and the indices are quotients of the average growth under some special experimentally arranged condition divided by an average growth under normally arranged condition.

After having become familiar with the above described technique, it seemed to us desirable to determine whether a simpler method might not give almost as satisfactory results, and thus be applicable in laboratories where the more elaborate apparatus is not available.

Methods

Transplants of actively growing fibroblasts were divided into an equal number of pieces, half of which were placed in Carrel flasks containing media representing an abnormal environment and half in a normal environment. The transplants were identified so that their origin could be traced. Their increment of growth was then estimated by one of us with the projectoscope, planimeter, adding machine and slide rule, according to Ebeling's technique. Simultaneously another observer estimated the increments of growth as follows: An eyepiece disc micrometer was placed on the diaphragm of a 10 × ocular which was used with an 180 mm. objective for the measurements of the transplants. The micrometer scale contained 100 equal divisions, with every tenth division numbered; and measurements were carried out to the nearest unit of 5. As the transplants originally were irregularly rectangular, the first measurement was made with the micrometer scale extending through the longest axis of the transplant; the ocular was then turned 90° and a second measurement was made through the other axis. If the transplant was very irregular in outline allowance was made so that approximately equal portions would extend outside and inside an imaginary line projected at right angles to the end of the axis being measured. On subsequent days as the growths assumed more and more a circular outline, the two axes were measured in the same manner. At the same time note was made of the qualitative character of the growth and the various elements composing it, and of the thickness of the culture by focusing at different levels.

The calculations of increments were made on the assumption that the areas occupied by the growths were perfectly circular, an assumption not in accord with the facts, but of sufficient validity so that the results might be tested against the more accurate data obtained with the projectoscope and planimeter. The average radius of any given lot of transplants was calculated as follows:

$$r = \frac{\text{Sum of all axes}}{4 \times \text{number of transplants}}.$$

The approximate average areas could be calculated from the formula πr^2; and from these areas the increments of growth could be determined. Comparative increments of growth, on the other hand, could be estimated directly from the squares of the radii as indicated in the formula $\frac{r_x^2 - r_o^2}{r_o^2}$ where r_o is the average radius of the original transplant, and r_x is the average radius on any given day. The effect of any given experimental environment, as in the case of the measurements made with the projectoscope, was calculated by the formula

$$\frac{\text{Increment under experimental environment}}{\text{Increment under normal environment}}.$$

RESULTS

The comparative results with the two methods are shown in Table I, where a surprisingly close agreement is seen in most instances, particularly in the case of older growths in which the new cells occupied a more nearly circular area than in the first 2 or 3 days after transplantation. A comparative index of more than 1 indicates that the experimental environment was more favorable than the normal one, and conversely an index of less than 1 shows the experimental environment was less favorable. In no case was the sign of the index different by the two methods; and in only part of one experiment (T-68) did the indices vary widely. In this case the observer was making his first ocular micrometric measurements; and the growths during the first few days showed marked irregularity so that calculations made on the assumption of a circular growth would not obtain. When, in the later days of the experiment, the growth became more circular and the observer more experienced, the indices agreed more closely. By calculating the initial areas of the transplants according to their true shape—square, triangular or circular—doubtless the two sets of figures could be brought into closer agreement.

In recording the rates of migration and growth of cells from explants of spleen and buffy coats of blood we have found the ocular micrometer especially useful, because the areas occupied by different types of cells could be more accurately measured on account of the possibility of

TABLE I

Experiment No	Part	Medium	Method of extraction		Day								
					1	2	3	4	5		7	8	9
T-64	*a*	Normal plasma	Projectoscope	Increment	0.6	3.1	8.1		12.0				
	b	Plasma removed	"	"	0.9	5.5	15.3		32.0				
				Ratio $\frac{a}{b}$	*0.66*	*0.56*	*0.53*		*0.37*				
	a	Normal plasma	Ocular micrometer	Increment		1.2	4.0	7.2					
	b	Plasma removed	" "	"		2.1	7.2	14.1					
				Ratio $\frac{a}{b}$		*0.58*	*0.53*	*0.52*					
T-66	*a*	Tb plasma	Projectoscope	Increment		1.2	3.6	8.6		16.7		32.2	
	b	Normal plasma	"	"		2.0	10.6	18.5		32.6		50.0	
				Ratio $\frac{a}{b}$		*0.6*	*0.36*	*0.46*		*0.5*		*0.65*	
	a	Tb plasma	Ocular micrometer	Increment		1.7	3.4	6.4		20.0			
	b	Normal plasma	" "	"		2.7	5.9	15.1		30.0			
				Ratio $\frac{a}{b}$		*0.63*	*0.58*	*0.36*		*0.66*			
T-67	*a*	Tb plasma	Projectoscope	Increment		0.58		2.3	5.0		11.3		
	b	Normal plasma	"	"		1.5		5.5	10.3		18.8		
				Ratio $\frac{a}{b}$		*0.39*		*0.42*	*0.48*		*0.6*		
	a	Tb plasma	Ocular micrometer	Increment		0.31	0.78		4.1		10.1		
	b	Normal plasma	" "	"		0.67	1.84		6.5		13.8		
				Ratio $\frac{a}{b}$		*0.46*	*0.42*		*0.63*		*0.73*		

T-68	*a*	Tb plasma	Projectoscope	Increment		1.5	6.1				30.6		49.5
	b	Normal plasma	"	"		0.8	2.8				13.5		11.0
				Ratio $\frac{a}{b}$		*1.86*	*2.2*				*2.3*		*2.9*
	a	Tb plasma	Ocular micrometer	Increment		1.83	7.25				26.2		46.0
	b	Normal plasma	" "	"		0.63	5.25				11.0		16.4
				Ratio $\frac{a}{b}$		*2.8*	*1.38*				*2.5*		*2.8*
T-71	*a*	Tb plasma	Projectoscope	Increment	0.45	2.1	4.2	6.5			21.2		41.0
	b	Normal plasma	"	"	0.38	2.1	7.4	8.5			24.4		45.6
				Ratio $\frac{a}{b}$	*1.18*	*1.0*	*0.57*	*0.76*			*0.89*		*0.9*
	a	Tb plasma	Ocular micrometer	Increment	0.6	2.6		7.4			16.2		27.0
	b	Normal plasma	" "	"	0.52	2.5		7.4			18.8		29.9
				Ratio $\frac{a}{b}$	*1.15*	*0.96*		*1.0*			*0.86*		*0.9*
T-67	*a*	Tb plasma	Projectoscope	Increment		0.58		2.3	5.0		11.3		
	c	Tb serum	"	"		1.5		8.3	16.5		40.0		
				Ratio $\frac{a}{c}$		*0.39*		*0.27*	*0.32*		*0.28*		
	a	Tb plasma	Ocular micrometer	Increment		0.31	0.78		4.1		10.1		
	c	Tb serum	" "	"		1.1	2.87		16.4		39.8		
				Ratio $\frac{a}{c}$		*0.28*	*0.27*		*0.25*		*0.25*		

obtaining a microscopic picture of each cell simultaneously with the measurements. The fact that these migrating cells quickly occupy a circular area also tends to make the two methods agree more closely than in the case of fibroblastic growths.

Another advantage of the ocular micrometric method is the possibility of placing the microscope in a warm box during the period of measurement, and thus one may avoid chilling the cultures and retarding their growth.

DISCUSSION

In presenting the ocular micrometer measurements as a basis for the calculation of growths of tissue cultures we do not mean to imply that it is as accurate as those given with the projectoscope and planimeter, nor do we think that the ocular micrometer has not been previously employed in examining tissue cultures, since remarks are encountered in the literature indicating that it has been used. In neither Fischer's book (2), in Levi's article on technique (3), nor elsewhere, have we found that approximate areas calculated from measurements of axes have been used for estimation of increments of growth or for comparative indices. While the simple inspection of growths will often give a fairly correct idea of the influence of a particular environment on the growth of cells *in vitro*, in many instances, where the culture media is composed of complex substances, it becomes necessary to keep quantitative records of the increment of growth if one is to estimate the effect of the components in the medium. The demands for quantitative data are even more pressing as one multiplies the number of pieces examined and the number of factors in the environment. Because the results of estimating the areas, and especially the comparative growth indices, were in such close agreement we feel that where the more complicated apparatus is not available one is justified in employing a method requiring simply an ocular micrometer and slide rule in addition to the usual material demanded for tissue cultures.

SUMMARY

In estimating the increments of growth of tissue cultures and of comparative indices it is possible to use areas calculated from measurement of two axes made with an ocular micrometer in an ordinary mi-

croscope. On the assumption that the growths are approximately circular in outline the squares of their radii are used as the factors for comparison. Results obtained with this method agree fairly closely with those derived from projectoscopic drawings and planimetric measurements.

BIBLIOGRAPHY

1. Ebeling, A. H., *J. Exp. Med.*, 1921, **34**, 231.
2. Fischer, A., Gewebezüchtung, Munich, Müller and Steinicke, 3rd edition, 1930.
3. Levi, G., Gewebezüchtung, in Péterfi, T., Methodik der wissenschaftlichen Biologie, Berlin, Julius Springer, 1928, **1**, 494.

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THE ANTIBODY RESPONSE OF RABBITS TO INJECTIONS OF EMULSIONS AND EXTRACTS OF HOMOLOGOUS BRAIN

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Since the discovery of hemolysins by Bordet attempts have been made to demonstrate antibodies against practically every type of cell and tissue in the body. Most of the efforts were prompted by the idea that certain diseases, for example nephritis and diabetes, characterized by degenerative changes in the affected organs, might be due to the development of antibodies against the tissues of the particular organ involved. However, the early claims made for the specificity of organ antibodies have been discounted to such an extent by later more seasoned work that the theories evolved from them have been discarded in the majority of instances. In the period since 1926, Brandt, Guth, and Müller (1) and others (2, 3, 4) have demonstrated that brain tissue contains an alcohol-soluble lipoid which functions as a haptene and that when this lipoid is mixed with a heterologous protein a complete antigen is formed capable of inciting in animals the development of complement-fixing antibodies which are organ- rather than species-specific. This discovery again opens, for brain at least, the question of the etiological rôle of organ-specific antibodies in certain degenerative diseases and strengthens materially the hypothesis that the encephalomyelitis which follows antirabies vaccination, if not the entire group of postinfection encephalitides, is in some manner associated with the development of specific antibodies for brain.

EXPERIMENTAL

Since a number of workers (2, 4) have already shown that fresh emulsions of homologous brain possess little or no antigenicity and that only when a heterologous protein is added to the brain haptene does it become a complete antigen, we were interested to see whether certain degenerative processes such as autolysis are capable of altering homologous brain material to such an extent that it becomes a complete antigenic complex. If this proved to be so, we planned to study the characteristics of the antigen as well as of the antibodies elicited by it. To this end rabbits were injected (1) with freshly prepared emulsions of rabbit brain, (2) with similar emulsions to which pig serum had been added, (3) with sterile emulsions of rabbit brain that had been allowed to stand at room temperature for 5 to 30 days, (4) with fresh emulsions prepared from rabbit brains experimentally infected with vaccine virus, and (5) with alcoholic extracts of rabbit brain plus pig serum. Following the injections sera of the animals were examined by means of complement fixation tests for the presence of antibodies against aqueous emulsions and alcoholic extracts of various organs.

Methods

Rabbits.—Only young mature rabbits averaging 2000 gm. in weight were used. To insure a maximum uniformity in the antibody response, only pure-strain self-blue English rabbits received injections. The rabbits which received injections of emulsions of brain infected with vaccine virus were immunized 2 weeks prior to the initiation of the experiment by means of intradermal injections of 0.25 cc. of a 1:10 dilution of vaccine virus prepared in tissue culture according to the method of Rivers (5).

Emulsions of Fresh Brain.—5 gm. of brain tissue removed aseptically from a rabbit were ground in a mortar with alundum until thoroughly macerated. 80 cc. of Locke's solution were added by increments during the process of grinding until the tissue was well emulsified. The suspension was then centrifuged at low speed for 2 minutes and the supernatant fluid removed by means of a pipette. The inoculum was prepared by the addition of 10 cc. of Locke's solution to each 40 cc. of the supernatant fluid.

Emulsions of Fresh Brain Plus Pig Serum.—Emulsions of fresh brain plus pig serum were prepared in a manner similar to that just described for the preparation of emulsions of fresh brain, except that in the final step, 10 cc. of pig serum, instead of Locke's solution, were added to each 40 cc. of the centrifuged suspension.

Emulsions of Autolyzed Brain.—Brain tissue was ground directly to a 5 per cent emulsion in a manner similar to that described for the preparation of emulsions of fresh brain. The supernatant fluid from the centrifuged suspension was transferred to a sterile flask that was then closed by a cotton plug over which were placed several layers of tin-foil. This material was allowed to stand at room temperature during the course of the experiment. As required for the injections, portions of the emulsion were removed from 5 to 30 days after preparation. Before use and from time to time during the experiment samples of the emulsion were tested for the presence of bacterial contaminants by means of aerobic and anaerobic cultures made in meat infusion broth (pH 7.8) and on blood agar. In no case during the course of an experiment were bacteria grown from emulsions found sterile at the start.

Emulsions of Brain Infected with Vaccine Virus.—A rabbit was inoculated intracerebrally with 0.25 cc. of a 5 per cent emulsion of rabbit brain infected with the Levaditi strain of vaccine virus. On the 3rd day after injection usually at the height of the infection the rabbit was killed. A 5 per cent emulsion in Locke's solution was prepared from the brain, the technique being similar to that described for the preparation of emulsions of fresh normal brain. The absence of ordinary bacterial contaminants in the brain was shown by means of aerobic and anaerobic cultures made in meat infusion broth (pH 7.8). On each occasion an additional rabbit was inoculated intracerebrally with a portion of the emulsion in order to furnish brain material for the next immunizing injection.

Alcoholic Extracts.—Alcoholic extracts of brain were prepared in the following manner: A known amount of tissue was thoroughly macerated in a mortar to which were added 5 cc. of 95 per cent ethyl alcohol for each gram of brain. The mixture was transferred to a flask which was then closed by a well fitting cork and allowed to stand at room temperature for 5 to 10 days. Then the material was passed through filter paper. In order to make immunizing emulsions 10 cc. of the filtrate were evaporated almost to dryness on the water bath and the residue was emulsified in 20 cc. of Locke's solution to which 5 cc. of pig serum had been added. For the experiments in which alcoholic extracts of organs other than brain were used the method of preparation of the extracts was the same.

Immunization of Rabbits.—Each rabbit received intraperitoneally 10 injections of 5 cc. of the brain emulsions or brain extracts. The inoculations were made at intervals of 2 or 3 days, and 10 days after the last injection the animals were bled from ear veins to furnish sera for the complement fixation tests.

Complement Fixation Test.—The antisheep-cell system was used in the complement fixation tests. 0.2 cc. of diluted inactivated antiserum from rabbits that had received the different preparations of brain, 0.2 cc. of antigen (organ emulsions or extracts), and 0.2 cc. of guinea pig serum diluted so as to contain 2 units of complement, were mixed in a Wassermann tube and incubated for 30 minutes at 37°C. in a water bath. 0.2 cc. of 5 per cent suspension of washed sheep cells and 0.2 cc. (2 units) of antisheep-cell amboceptor were added and the tubes again incubated for 30 minutes at 37°C. The results of the tests were recorded in terms of the amount of fixation of complement, from 4+ indicating total complement fixation and no hemolysis to – designating complete laking of the sheep cells. Two types of antigen were used in the tests, aqueous emulsions and alcoholic extracts. In order to make aqueous antigens 1 gm. of the particular organ desired was thoroughly ground in a mortar with alundum, and 25 cc. of physiological saline solution were added by increments during the process of grinding until a homogeneous emulsion resulted. This suspension was filtered through a tightly folded pad of absorbent cotton to remove the alundum and large bits of tissue. When less concentrated emulsions were used, the necessary dilutions were made from the original 1:25 suspension. The preparation of alcoholic antigens was accomplished by the evaporation of a known volume of the alcoholic organ extract almost to dryness on a water bath and the suspension of the residue in a given volume of physiological saline solution. The concentration of this antigen was designated by the ratio between the original volume of alcoholic extract used and the amount of saline solution in which the residue was resuspended.

TABLE I

Summary of Results of Complement Fixation Experiments Indicating the Presence or Absence of Antibodies in the Sera of Rabbits Injected with Various Emulsions and Extracts of Homologous Brain

Material used for immunization	Amount of complement fixed by different antisera in the presence of an aqueous emulsion of rabbit brain					
	Dilution of serum					
	1:5	1:10	1:20	1:40	1:80	1:160
Fresh rabbit brain	–	–	–	–	–	–
" " " plus pig serum	4+	4+	2+	–	–	–
Autolyzed rabbit brain	4+	4+	4+	4+	2+	–
Vaccine virus rabbit brain	4+	4+	4+	4+	1+	–
Alcoholic extract of rabbit brain plus pig serum	4+	4+	1+	–	–	–

4+ indicates complete fixation of complement.
– indicates no fixation.

The results given for alcoholic extract of rabbit brain plus pig serum were taken from another experiment and are therefore not strictly comparable to the others.

Antigenicity of Homologous Brain

In order to test the antigenic qualities of homologous brain six experiments were performed, in which 79 rabbits were used. The animals in each experiment were divided into groups of four or five, and each group of animals received injections of one of the preparations of homologous brain tissue described above. For the most part each experiment was a repetition in whole or in part of the others. The results obtained in a typical experiment have been summarized in Table I and clearly show (1) that rabbits injected with fresh emulsions of homologous brain developed few or no antibodies against brain tissue, (2) that antibrain antibodies were elicited by fresh emulsions of homologous brain to which pig serum had been added, (3) that autolysis alone changed the nature of homologous brain tissue to such an extent that it became a complete antigen, (4) that rabbits which received injections of emulsions of fresh homologous brain infected with vaccine virus readily developed antibrain antibodies, and (5) that antibodies against brain tissue appeared in the serum of rabbits injected with emulsions of alcoholic extracts of rabbit brain to which pig serum had been added.

TABLE II

Summary of Results of Complement Fixation Tests Conducted with Sera of Rabbits Immunized with Autolyzed Homologous Brain Emulsions

Antigen	Dilution of antigen	Dilution of serum							
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640
Emulsion of rabbit brain	1:50	4+	4+	4+	4+	4+	4+	3+	–
" " " kidney	1:50	4+	4+	3+	–	–	–		
" " " liver	1:50	4+	1+	–	–	–	–		
" " " spleen	1:50	3+	–	–	–	–	–		
" " " red cells	1:25	–	–	–	–	–	–		
" " guinea pig brain	1:50	4+	4+	4+	4+	4+	4+	1+	–
" " " " kidney	1:50	4+	4+	2+	–	–	–		
" " " " liver	1:50	4+	4+	1+	–	–	–		
" " " " spleen	1:50	4+	1+	–	–	–	–		
" " " " red cells	1:25	–	–	–	–	–	–		
Alcoholic extract of rabbit brain	1:2	4+	4+	3+	–	–	–		
" " " " kidney	2:1	–	–	–	–	–	–		
" " " " liver	2:1	–	–	–	–	–	–		
" " " " spleen	1:2	–	–	–	–	–	–		

4+ indicates complete fixation of complement.

– indicates no fixation.

When aqueous emulsions were used as antigens in the tests lack of organ specificity is evident. Organ specificity is obvious in the tests in which alcoholic extracts were used.

Specificity of the Antiserum

Having demonstrated that autolytic or disease processes acting on brain tissue can cause it to become fully antigenic for homologous animals, we proceeded to determine whether the antibodies elicited by such altered brain material are specific for brain. For this purpose, an antiserum prepared in rabbits by means of injections of emulsions of autolyzed brain tissue was subjected to complement fixation tests in which aqueous emulsions of rabbit brain, kidney, liver, spleen, and red blood cells as well as emulsions of similar organs of the guinea pig were used as antigens. In addition, the antiserum was tested against alcoholic extracts of various rabbit organs. The results of one experiment are shown in Table II. The antiserum reacted most strongly with rabbit and guinea pig brain, yet some antibodies were also present against the tissues of practically all the other organs of the rabbit and guinea pig that were tested. When alcoholic extracts were used as antigens in the complement fixation tests, however, the antiserum was organ-specific (brain-specific) within the range of antigens used. Several workers (3, 6, 7, 8) have reported similar results for antisera prepared by the immunization of animals with emulsions of heterologous brain. Further tests for organ specificity were made on antisera which had been prepared by the injection into rabbits of alcoholic extracts of homologous brain plus pig serum. The results, summarized

TABLE III

Summary of Results of Complement Fixation Tests Conducted with Sera of Rabbits Immunized with Alcoholic Extracts of Rabbit Brain Plus Pig Serum

Antigen	Dilution of antigen	Dilution of serum					
		1:5	1:10	1:20	1:40	1:80	1:160
Emulsion of rabbit brain	1:50	4+	4+	4+	3+	1+	–
“ “ “ kidney	1:50	2+	–	–	–	–	–
“ “ “ liver	1:50	–	–	–	–	–	–
“ “ “ spleen	1:50	–	–	–	–	–	–
“ “ guinea pig brain	1:50	4+	4+	4+	2+	–	–
“ “ “ “ kidney	1:50	3+	–	–	–	–	–
“ “ “ “ liver	1:50	–	–	–	–	–	–
“ “ “ “ spleen	1:50	1+	–	–	–	–	–

4+ indicates complete fixation of complement.

– indicates no fixation.

The results of the complement fixation tests show almost complete organ specificity. Compare with results shown in Table II.

in Table III, show that such sera were almost completely organ-specific. The minor fixations of complement in the presence of emulsions of other organs were overshadowed by the high titer of the antisera in the presence of brain antigen. This quantitative organ specificity of antisera prepared by injections of alcoholic extracts of brain has been noted by a number of investigators (9, 10).

When one considers the complex nature of the emulsions of brain with which the rabbits were immunized, a possible cause becomes apparent for the lack of specificity evidenced by the antibodies when they were tested for ability to fix complement in the presence of aqueous emulsions of different organs. The brain undoubtedly contains certain tissues which are common to other organs. For lack of more exact knowledge we shall consider the common factor to be connective tissue. Rabbits immunized with emulsions of brain therefore develop antibodies against connective tissue as well as against the specific brain antigen. Since the connective tissue antigen apparently is not as alcohol-soluble as is the antigen of brain, complement fixation tests done with alcoholic antigens show more pronounced organ specificity. If this reasoning is correct, the serum of rabbits immunized against emulsions of brain must contain at least two, perhaps more, kinds of antibodies, one specific for brain antigen and another which is not organ-specific. A similar theory for the lack of specificity in antisera prepared in animals by the injections of emulsions of heterologous organs has already been advanced by Fleisher and his co-workers (6, 7).

Absorption of Antibodies

In order to determine whether non-specific as well as specific antibodies were present in the antibrain sera a number of absorption experiments were performed.

Method of Absorption.—A 1:25 emulsion, in physiological saline solution, of the desired organ was made in a manner similar to that used for the preparation of antigens for complement fixation tests. The technique of this procedure has already been described. The organ emulsion was then added to an equal volume of a 1:2.5 dilution, in physiological saline solution, of the antiserum, and the mixture was heated at 56°C. for 2 hours in the water bath. After standing overnight at room temperature it was centrifuged at high speed for half an hour, and the supernatant liquid was recovered with a pipette. In order to remove all of the antigen-antibody complex, however, it was necessary to pass the mixture through a Seitz filter. As a control a second sample of the same antiserum was subjected to similar manipulations with the exception that physiological saline solution was added to it instead of an absorbing antigen. Experiments were also carried out in which an alcoholic extract of brain was used as an absorbing agent. The technique was similar to that just described except that a 1:16 dilution of the alcoholic antigens was found to yield maximum absorption.

TABLE IV

Summary of Results of Complement Fixation Tests Conducted with Antibrain Serum before and after Absorption with Aqueous Emulsions of Rabbit Kidney

Serum	Antigen	Dilution of antigen	Dilution of serum					
			1:5	1:10	1:20	1:40	1:80	1:160
Unabsorbed	Emulsion of rabbit brain	1:600	4+	4+	3+	1+	–	–
	“ “ “ kidney	1:50	4+	4+	3+	–	–	–
	“ “ “ liver	1:50	4+	1+	–	–	–	–
	“ “ “ spleen	1:50	3+	–	–	–	–	–
	“ “ guinea pig brain	1:600	4+	4+	4+	2+	–	–
	“ “ “ “ kidney	1:50	4+	4+	2+	–	–	–
	“ “ “ “ liver	1:50	4+	4+	1+	–	–	–
	“ “ “ “ spleen	1:50	4+	1+	–	–	–	–
Absorbed with aqueous emulsion of rabbit kidney	Emulsion of rabbit brain	1:600	4+	4+	2+	–	–	–
	“ “ “ kidney	1:50	–	–	–	–	–	–
	“ “ “ liver	1:50	–	–	–	–	–	–
	“ “ “ spleen	1:50	–	–	–	–	–	–
	“ “ guinea pig brain	1:600	4+	4+	3+	2+	–	–
	“ “ “ “ kidney	1:50	–	–	–	–	–	–
	“ “ “ “ liver	1:50	–	–	–	–	–	–
	“ “ “ “ spleen	1:50	–	–	–	–	–	–

4+ indicates complete fixation of complement.

– indicates no fixation.

The results of the absorption experiments show that an emulsion of rabbit kidney removed the non-specific antibodies from the antibrain serum.

Three kinds of absorption experiments were conducted. In the first, antibrain sera were absorbed with emulsions of rabbit kidney which, containing only non-specific antigen, should not remove specific antibodies for brain. In the second, emulsions of rabbit brain which contain both the specific and non-specific antigens were used for absorption. In this case both kinds of antibodies should be removed from the antisera. And finally antibrain sera were absorbed with alcoholic extracts of rabbit brain which contain only or principally the specific antigen and should therefore remove from the sera only the specific antibodies for brain. The results of these absorption experiments are summarized respectively in Tables IV, V, and VI. From the results shown in Table IV it can be seen that an emulsion of rabbit kidney absorbed the non-specific antibodies from the serum so that it failed to react to any organ emulsion except that prepared from brain. When the absorption was carried out with an emulsion of rabbit brain (Table V) which contains both the specific and non-specific antigens, both kinds of antibodies were removed and the serum failed to fix comple-

TABLE V

Summary of Results of Complement Fixation Tests Conducted with Antibrain Serum before and after Absorption with Aqueous Emulsions of Rabbit Brain

Serum	Antigen	Dilution of antigen	Dilution of serum					
			1:5	1:10	1:20	1:40	1:80	1:160
Unabsorbed	Emulsion of rabbit brain	1:600	4+	4+	4+	3+	2+	1+
	“ “ “ kidney	1:50	4+	4+	3+	–	–	–
	“ “ “ liver	1:50	4+	1+	–	–	–	–
	“ “ “ spleen	1:50	3+	–	–	–	–	–
	“ “ guinea pig brain	1:600	4+	4+	4+	2+	–	–
	“ “ “ “ kidney	1:50	4+	4+	2+	–	–	–
	“ “ “ “ liver	1:50	4+	4+	1+	–	–	–
	“ “ “ “ spleen	1:50	4+	1+	–	–	–	–
	Alcoholic extract of rabbit brain	1:2	4+	4+	3+	–	–	–
Absorbed with aqueous emulsion of rabbit brain	Emulsion of rabbit brain	1:600	–	–	–	–	–	–
	“ “ “ kidney	1:50	–	–	–	–	–	–
	“ “ “ liver	1:50	–	–	–	–	–	–
	“ “ “ spleen	1:50	–	–	–	–	–	–
	“ “ guinea pig brain	1:600	–	–	–	–	–	–
	“ “ “ “ kidney	1:50	–	–	–	–	–	–
	“ “ “ “ liver	1:50	–	–	–	–	–	–
	“ “ “ “ spleen	1:50	–	–	–	–	–	–
	Alcoholic extract of rabbit brain	1:2	–	–	–	–	–	–

4+ indicates complete fixation of complement.

– indicates no fixation.

The results of the absorption experiments show that an emulsion of brain removed both the specific and non-specific antibodies from the antibrain serum.

ment in the presence of any of the organ emulsions. Finally, absorption of the antiserum with an alcoholic extract of rabbit brain (Table VI) resulted principally in the removal of antibodies against brain, permitting the serum still to react with emulsions of all the organs which contained the non-specific antigen. One might wonder why the antiserum absorbed with the alcoholic extracts of brain did not react to a slight extent with emulsions of brain as well as with emulsions of other organs because brain tissue contains both specific and non-specific antigens. This is explained by the fact that in order not to mask the sensitivity of the complement fixation test by a large amount of antigen, it was necessary to use a 1:600 dilution of the emulsion of brain. At this concentration the non-specific antigen contained in brain tissue was diluted beyond its power to fix complement in the presence of the antiserum. The results of the absorption experiments just enumerated appear to indicate that two if not more antigen-antibody systems were involved, one of which was specific for brain while

TABLE VI

Summary of Results of Complement Fixation Tests Conducted with Antibrain Serum before and after Absorption with Alcoholic Extract of Rabbit Brain

Serum	Antigen	Dilution of antigen	Dilution of serum					
			1:5	1:10	1:20	1:40	1:80	1:160
Unabsorbed	Alcoholic extract of rabbit brain	1:2	4+	4+	4+	2+	1+	–
	Emulsion of rabbit brain	1:600	4+	4+	3+	2+	–	–
	" " " kidney	1:50	4+	4+	2+	–	–	–
	" " " liver	1:50	4+	3+	1+	–	–	–
	" " " spleen	1:50	4+	1+	–	–	–	–
Absorbed with alcoholic extract of rabbit brain	Alcoholic extract of rabbit brain	1:2	–	–	–	–	–	–
	Emulsion of rabbit brain	1:600	–	–	–	–	–	–
	" " " kidney	1:50	4+	4+	2+	–	–	–
	" " " liver	1:50	4+	2+	–	–	–	–
	" " " spleen	1:50	3+	–	–	–	–	–

4+ indicates complete fixation of complement.

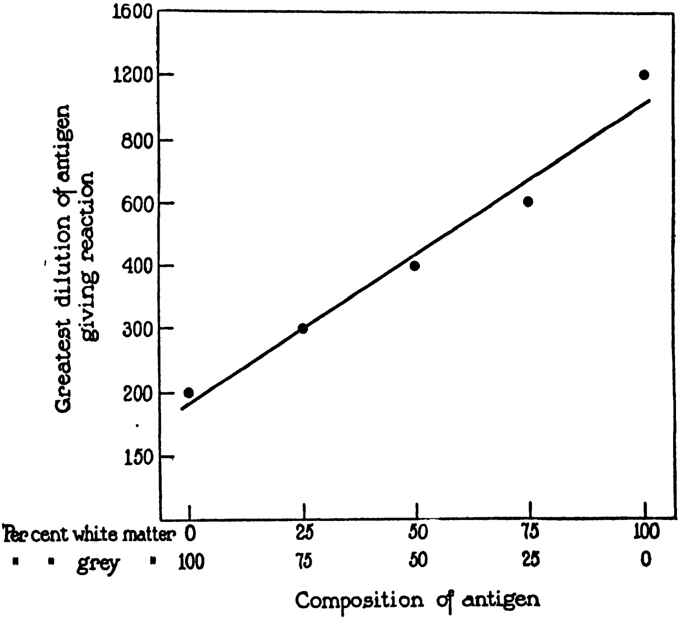
– indicates no fixation.

The results of the absorption experiments show that an alcoholic extract of brain removed principally the antibrain antibodies from the antibrain serum.

the other was associated with some element or elements common to many organs.

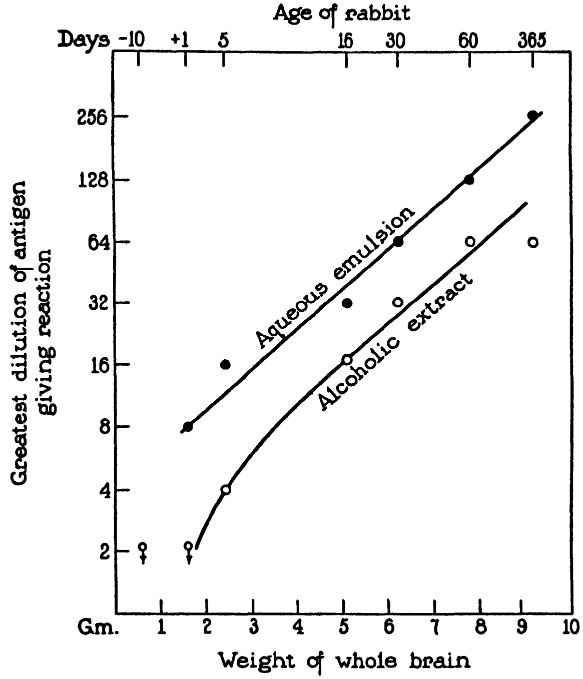
Nature of the Antigen

Having satisfied ourselves that emulsions of homologous brain can be made to become antigenic under certain conditions we were interested to determine what substance in the brain causes it to act as a specific antigen. It occurred to us that the specificity might be associated in some manner with myelin. The most obvious way of testing such an idea was to find out whether the amount of myelin in brain antigens used for fixation of complement in the presence of antibrain sera runs parallel to the degree of fixation. A rabbit brain was therefore divided as nearly as possible into two portions, white matter which contains large numbers of myelinated nerve fibers and grey



TEXT-FIG. 1. The graph represents the relative antigenicity of white and grey matter of brain. The greatest dilution of each mixture of antigens which fixed complement in the presence of an antibrain serum has been plotted against the composition of the mixtures of white and grey matter.

matter which has a lower myelin content. From the two portions, aqueous emulsions were made, after which five mixtures were prepared by the combination of different quantities of them so that the ratio of grey emulsion to white emulsion varied from 100 per cent white to 100 per cent grey. The antigenicity of each of the emulsions was determined quantitatively in complement fixation tests conducted with antibrain serum. Text-fig. 1 shows the results graphically. It is apparent from them that the antigenicity of the white matter was roughly six times stronger than that of the grey matter, a property which at least paralleled the content of myelin in the two emulsions.



Text-Fig. 2. The graph represents the relative antigenicity of brains of rabbits of different ages. The greatest dilution of antigen which fixed complement in the presence of an antibrain serum has been plotted as a function of the weights of the whole brain from which the antigen was made. The ages of the rabbits from which the brains were removed is also indicated. The ordinates for the curve for aqueous antigens are plotted as dilutions of a 1:50 brain emulsion; *i.e.*, a 1:2 dilution in the graph is actually a 1:100 emulsion of brain tissue.

Further investigation of the relation between the myelin content and the antigenicity of brain tissue was made possible by the fact that myelin is practically absent from the central nervous system of fetal or newly born animals and increases with the age of the animal. We, therefore, prepared emulsions of the brain tissue of rabbits of different ages, ranging from the fetal stage to maturity. The relative antigenic properties of these emulsions were determined by the fixation of complement in the presence of antibrain serum. Furthermore, in order to eliminate as far as possible the non-specific antigen, alcoholic extracts were made from portions of the brains of these animals and tested for antigenicity. In Text-fig. 2 the results of such an experiment are shown graphically. It is immediately apparent that in newly born rabbits the content of organ-specific antigen in the brain emulsions was negligible and that it increased steadily with the age of the animal to reach a high concentration at maturity. Alcoholic extracts of brains of fetal or newly born rabbits failed to fix complement in the greatest concentration used, while extracts of brains of adult rabbits contained sufficient antigen to fix complement in high dilutions.

Observations on the Clinical and Pathological Findings in Rabbits Injected with Emulsions and Extracts of Homologous Brain

The rabbits which received injections of the different emulsions or extracts of brain were carefully observed during and after the course of immunization for the appearance of abnormalities attributable to the procedures used. In no instance was a reaction seen immediately following primary injection. For the most part the rabbits remained in good condition throughout the experiments, although some that received injections of emulsions of autolyzed brain lost weight during the course of the experiments. In every experiment, however, a certain number of rabbits developed signs referable to the central nervous system. The course in every case was the same: the rabbits developed a slight weakness of the hind legs which progressed rapidly to a complete paralysis of the hind quarters and was accompanied by a progressive emaciation; retention of urine was noted; and death occurred after an illness of a few days.

At necropsy the central nervous system and other organs in the gross were normal in appearance. In the brains of some of the paralyzed rabbits stained sections showed perivascular infiltration and small areas of necrosis surrounded by zones of inflammation, in others evidences of healing lesions were seen, in still others no pathological changes were noted. Demyelination was not observed in any instance in the brain and cord, and the peripheral nerves appeared to be normal. Lesions identical in appearance with those just described were also found in some of the non-paralyzed rabbits sacrificed as controls and were considered to be due to the activity of *Encephalitozoon cuniculi*. The fact that the lesions were not present in all of the paralyzed animals and that they occurred in some of the non-paralyzed rabbits led us to conclude that they should not be looked upon as the cause of the paralyses. This opinion is substantiated by the observations of Hurst (11), who, working with rabbits known to be free from *Encephalitozoon* infection, found no pathological changes in the central nervous system of animals which became paralyzed following injections of emulsions of heterologous brain.

TABLE VII

Relation between the Number of Paralyzed Rabbits and the Immunizing Materials

Material used for immunization	No. of injections	No. of rabbits injected	No. paralyzed	Per cent paralyzed
Fresh rabbit brain	5–12	19	0	0
" " " plus pig serum	5–10	14	1	7
Alcoholic extract of rabbit brain plus pig serum	13–26	8	1	12
Vaccine virus rabbit brain	5–12	10	1	10
Autolyzed rabbit brain	5–15	28	9	32

It is interesting to note that the incidence of paralysis in the rabbits of the different groups paralleled the antigenicity of the brain emulsions and extracts that they received. In Table VII, the different immunizing emulsions and extracts are listed in the order of their antigenicity and the incidence of paralysis caused by them. None of the rabbits that received fresh emulsions of homologous brain, which has little or no antigenicity, became paralyzed, while 9 of 28 rabbits (32 per cent) injected with autolyzed homologous brain, a good antigen, showed evidences of paralysis. These observations, however, cannot be considered evidence that the paralysis was directly related to the antigenicity of the brain emulsions, because Hurst (11) has pointed out that similar paralytic accidents follow injections of substances other than brain and also because the paralysis might have been induced by some toxic substance in the autolyzed or diseased brain tissue.

DISCUSSION

It has been shown by a number of investigators that heterologous brain (2, 3), alcoholic extracts of heterologous brain plus pig serum (1, 10), homologous brain plus pig serum (4), and alcoholic extracts of homologous brain plus pig serum (10) excite in animals receiving them the production of specific antibrain antibodies capable of demonstration either by means of complement fixation tests or by precipitin reactions. In addition to this we have been able to demonstrate that homologous brain altered by autolysis alone or by infection with vaccine virus alone becomes antigenic and is then capable of inducing the production of antibodies specific for brain tissue.[1] Furthermore, we have been able to show that the specific antigenicity of homologous brain runs parallel to the myelin content of the tissue.

In view of the facts just enumerated it is interesting to speculate about the etiology of certain diseases of the central nervous system, particularly the demyelinating maladies, such as the postinfection encephalitides, multiple sclerosis, Schilder's disease, and the encephalomyelitis occurring after antirabies vaccination. Speculation would be more interesting if it were possible to produce demyelination in animals by means of injections of brain tissue or if it were possible to demonstrate either brain antigen or antibrain antibodies in the blood of patients with demyelinating diseases. Hurst (11) from his review of the literature dealing with the effects on animals of repeated injections of brain tissue and from the results of his own experiments concluded that, although paralysis has been observed following injections of emulsions of brain, no one has definitely shown the presence of demyelination in the brains and cords of the paralyzed animals. Rivers,

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[1] After our work was completed, Lewis (13) reported that antibrain sera are also specific for testicular tissue. We did not examine our antibrain sera by means of complement fixation tests in which testicular tissue was used as an antigen. Consequently we have no information regarding this matter.

Sprunt, and Berry (12), however, recorded the fact that two of eight monkeys which received repeated injections of emulsions and alcoholic extracts of rabbit brain developed paralysis associated with demyelination. In regard to the presence of brain antigen or antibrain antibodies in the blood of patients with demyelinating diseases no significant reports have been seen. Although speculation about the relation of antibrain antibodies to certain demyelinating maladies is extremely intriguing, further facts must be obtained before a reasonable hypothesis concerning the matter can be presented.

SUMMARY

Rabbits injected with fresh emulsions of homologous brain developed few or no antibodies capable of fixing complement in the presence of aqueous emulsions or alcoholic extracts of rabbit brain. Complement-fixing antibodies, however, were produced in rabbits by means of injections (1) of sterile emulsions of homologous brain which had been allowed to stand at room temperature for 5 to 30 days and (2) of emulsions of homologous brain experimentally infected with vaccine virus. The antisera that were produced following injections of emulsions of autolyzed homologous brain were shown by absorption tests to contain both specific and non-specific antibodies. The specific brain antigen was found to be approximately six times as abundant in the white matter as in the grey. It was almost absent from the brain of fetal and newly born rabbits, but increased in amount with the age of the animal to reach a maximum concentration at maturity. The specific antigen seemed to parallel the myelin content of brain tissue.

BIBLIOGRAPHY

1. Brandt, R., Guth, H., and Müller, R., *Klin. Woch.*, 1926, **5**, 655.
2. Witebsky, E., and Steinfeld, J., *Z. Immunitätsforsch.*, 1928, **58**, 271.
3. Plaut, F., and Kassowitz, H., *Z. Immunitätsforsch.*, 1929, **63**, 428.
4. Lewis, J. H., *J. Immunol.*, 1933, **24**, 193.
5. Rivers, T. M., *J. Exp. Med.*, 1931, **54**, 453.
6. Fleisher, M. S., and Arnstein, N., *J. Immunol.*, 1921, **6**, 223.
7. Fleisher, M. S., *J. Immunol.*, 1922, **7**, 51.
8. Moran, F., *Z. Immunitätsforsch.*, 1930, **67**, 115.
9. Weil, A. J., *Z. Immunitätsforsch.*, 1928, **58**, 172.
10. Heimann, F., and Steinfeld, J., *Z. Immunitätsforsch.*, 1928, **58**, 181.
11. Hurst, E. W., *J. Hyg.*, 1932, **32**, 33.
12. Rivers, T. M., Sprunt, D. H., and Berry, G. P., *J. Exp. Med.*, 1933, **58**, 39.
13. Lewis, J. H., *J. Immunol.*, 1934, **26**, 331.

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STUDIES OF ACIDOSIS

XXII. APPLICATION OF THE HENDERSON-HASSELBALCH EQUATION TO HUMAN URINE*

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The Henderson (1909–10) equation

$$[\mathrm{H}^+] = K' \frac{[\mathrm{Ha}]}{[\mathrm{Ba}]} \tag{1}$$

relating hydrion concentration in buffer acid solutions to the ratio, free acid:alkali salt, has been frequently applied to the calculation of pH from H_2CO_3 and $BHCO_3$ in the blood, particularly since Hasselbalch (1916) transformed the equation for this purpose into logarithmic forms.

$$\mathrm{pH} = \mathrm{pK}' + \log \frac{[\mathrm{BHCO_3}]}{[\mathrm{H_2CO_3}]} \tag{2}$$

or

$$\mathrm{pH} = \mathrm{pK}' + \log \frac{[\mathrm{CO_2}] - 0.0591\alpha p}{0.0591\alpha p} \tag{3}$$

$[BHCO_3]$, $[H_2CO_3]$, and $[CO_2]$ indicate millimolar concentrations of bicarbonate, free carbonic acid, and total CO_2 respectively; α is the Bunsen solubility coefficient of CO_2; p is the CO_2 tension in mm. of mercury; $0.0591\ \alpha p = [H_2CO_3]$; pK′ is the negative logarithm of the apparent first dissociation constant of carbonic acid.

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* A preliminary communication concerning this work was delivered at the XIVth International Physiological Congress at Rome (Sendroy, Seelig, and Van Slyke, 1932).

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The accuracy with which the equation can be applied depends upon the exactness with which the constants, pK′ and α, are known for the particular solution investigated. For serum a number of authors have determined the value of pK′, and the results have been summarized by Hastings, Sendroy, and Van Slyke (1928), who found the value to be 6.10 ± 0.03. The value of α for normal serum has also been determined and found to be quite constant at 0.510, 93.4 per cent of the value in water (Van Slyke, Sendroy, Hastings, and Neill, 1928).

For studies of urine, on the other hand, there has been relatively little application of this equation. Gamble (1922), who used Equation 2 to calculate H_2CO_3 content from urine pH and CO_2 content, appears to be the first author to apply it to a physiological investigation. He used the same value for pK′, 6.1, which had been found for serum. He did not calculate CO_2 tension values, and hence did not require a value for α. Mainzer and Bruhn (1931) later determined α and pK′ values in twenty urine specimens, and found such wide variations in these values that accurate application of the Henderson-Hasselbalch equation to urine by the use of average α and pK′ values appeared to be impossible. Mainzer and Bruhn, for their pK′ calculations, determined pH with the quinhydrone electrode. Their α values were determined by a technique modeled in most details after that used by Van Slyke, Sendroy, Hastings, and Neill (1928). Mainzer and Bruhn's values of α and pK′ have thus far remained the only ones in the literature.

In the present paper we have redetermined the values of α and pK′ for human urine, with precautions outlined below. The pK′ values have been determined with the standard hydrogen electrode.

EXPERIMENTAL

The Bunsen solubility coefficient, α, was obtained by practically the same procedure used by Van Slyke, Sendroy, Hastings, and Neill (1928), in estimating it for serum, except that in most of the present determinations an analyzed mixture of CO_2 and air was used as the saturating gas instead of pure CO_2. The same results were obtained by both procedures. The CO_2 content of the air-CO_2 mixtures, at the end of saturation, was measured by analysis, either in the Haldane apparatus or in the manometric apparatus according to Van Slyke, Sendroy, and Liu (1932). Before saturation, the urines were acidified with HCl to a pH below 3.5. The applicability of HCl, and its relatively slight effect on α, have been discussed on p. 783 of Van Slyke, Sendroy, Hastings, and Neill (1928). Values of α were calculated according to Equation 4 (Equation 2 of the latter authors).

$$\alpha = \frac{[CO_2]}{100} \times \frac{760}{p} \times \frac{V_R}{V_{38^\circ}} = \frac{[CO_2]}{p} \times 7.57 \tag{4}$$

V_R and V_{38° represent respectively the volumes occupied by 1 gram of the solution at room temperature, when analyzed, and at 38°, the temperature of saturation. The factor 7.57 includes the value of V_R/V_{38° when the room temperature is 24–27°. Solubility coefficients calculated by Equation 4 were subsequently corrected for the slight effect of the added HCl.

The value of pK′ was obtained essentially by the procedure applied by Hastings, Sendroy, and Van Slyke (1928) for determination of pK′ in serum. For each experiment, enough urine for several analyses and fillings of the electrode vessel was saturated at 38° with a mixture of CO_2 and H_2. After saturation was complete, a portion of the gas mixture was analyzed for CO_2. The rest of the same gas mixture was then used in the pH determinations made in the Clark-Cullen electrode vessel. Duplicate pH values uniformly agreed within 0.005 unit. The CO_2 contents of the saturated urines were determined with the Van Slyke-Neill apparatus as described on p. 299 of Peters and Van Slyke (1932). Samples of appropriate size were taken to secure the maximum accuracy for values of $[CO_2]$ of 1 part in 300.

Total base was determined in the urine by a modification of Fiske's (1922) method. The phosphates were removed by Hoffman's procedure (1931), the bases were ashed to sulfates, and the SO_4 was weighed as $BaSO_4$. Enough urine, 10 or 15 cc., was used to yield 0.2 or 0.3 gram of $BaSO_4$.

Specific gravity readings were made with an ordinary bulb hydrometer. The gravities were read to 0.001, and are thus recorded, although calibration of the instrument against determinations by weight indicated its accuracy to be limited to 0.003.

RESULTS

Solubility of CO_2 in Urine at 38°

Our twenty-two values for α in Table I vary from 0.536 to 0.498, with an average of 0.522 ± 0.008. Figs. 1 and 2 show the decrease in α with increasing total base content and specific gravity. The dashed lines in Fig. 1 indicate the solubilities which Van Slyke, Sendroy, Hastings, and Neill (1928) found in aqueous chloride

TABLE I

Values of CO_2 Solubility Coefficient in Human Urine at 38°

Urine No.	Total base	Specific gravity	CO_2 tension at 38°	CO_2 content at 24-27°	α*
	m.-eq. per l.		*mm.*	*vol. per cent*	*cc. CO_2 per cc. solution*
1	255	1.030	91.1	6.28	0.524
2	246	1.024	122.3	8.08	0.504
3	63	1.008	100.7	7.11	0.536
4	247	1.031	109.6	7.24	0.503
5	254	1.035	710.8	46.48	0.498
6	193	1.018	110.6	7.60	0.524
7	68	1.011	109.2	7.56	0.528
8	76	1.012	107.0	7.34	0.523
9	140	1.023	109.5	7.62	0.531
10	222	1.026	111.2	7.51	0.515
11	93	1.009	110.8	7.74	0.532
12	151	1.013	111.3	7.59	0.520
13	79	1.007	108.6	7.57	0.530
14	54	1.019	107.0	7.43	0.529
15	157	1.010	105.2	7.27	0.527
16	176	1.019	110.1	7.50	0.518
17	109	1.025	109.9	7.64	0.530
18	136	1.019	107.0	7.34	0.522
19	125	1.015	108.9	7.49	0.524
20	95	1.020	108.9	7.56	0.530
21		1.017	107.6	7.24	0.513
22	60	1.010	105.7	7.39	0.532
Average..					0.522

* Corrected for effect of added HCl.

and acid phosphate solutions. Most of the urine values fall between those for solutions of KCl and NaH_2PO_4 of the same total base concentration. Urea, the only non-electrolyte normally present in sufficient concentration to affect α significantly, in maximal concentration (0.5 M or 3 per cent) lowers α by only 0.008

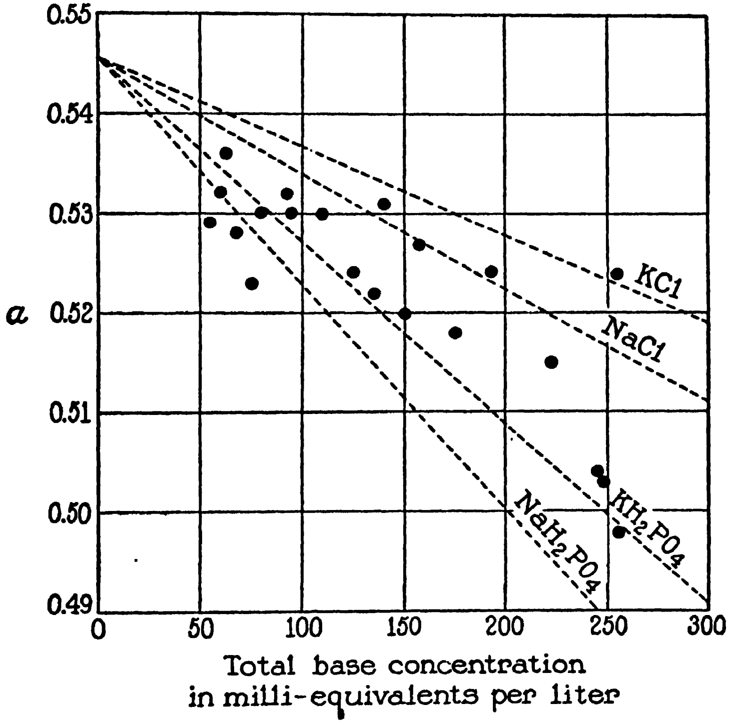

Fig. 1. The relationship between the CO_2 solubility coefficient of urine and its salt content. All points are for urine. Dotted lines are for salts, taken from Van Slyke, Sendroy, Hastings, and Neill (1928).

unit (Usher, 1910). Hence it is to be expected that the lowering of α of urine below that of water is chiefly a function of the salts, of which ordinarily the most abundant are chloride and acid phosphate.

Mainzer and Bruhn's (1931) values of α for twenty urines extend from 0.441 to 0.514, with an average of 0.482. Their average is 8 per cent below ours, and a number of their values are lower than any we obtained in the most concentrated urines.

Mainzer and Bruhn's technique, like ours, followed in general that of Van Slyke, Sendroy, Hastings, and Neill (1928); but Mainzer and Bruhn introduced the following changes first. They kept their samples under mineral oil saturated with the equilibrating gas mixture of CO_2 and air, instead of confining them in closed containers over mercury. Second, in transferring their samples of urine to the manometric apparatus, they apparently used open pipettes instead of the special closed pipette described in Section XII of Van Slyke and Neill (1924). It is possible that these differences in technique may have caused CO_2 losses and the comparatively low results of Mainzer and Bruhn.

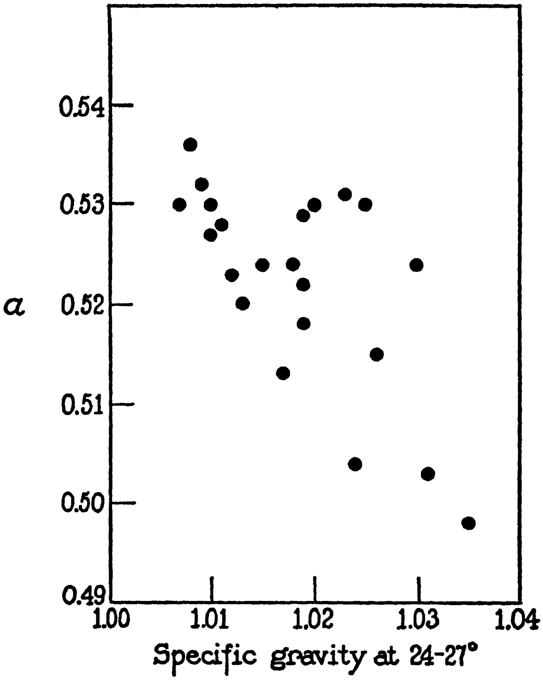

FIG. 2. The relationship between the CO_2 solubility coefficient of urine and its specific gravity.

pK′ of Urine at 38°

Our data in Table II give pK′ values in urine averaging 6.10 ± 0.07. The maximum deviation from the average value is ±0.13.

TABLE II

*Values of pK′ in Human Urine at 38°**

Urine No.	CO_2 content analyzed (a)	CO_2 tension analyzed (b)	$[H_2CO_3]$ from individual α and (b) (c)	$[BHCO_3]$ from (a) − (c) (d)	E corrected to 760 mm. H_2 pressure (e)	E_0 (f)	pH determined (g)	pK′ (h)
	mM per l.	*mm.*	*mM per l.*	*mM per l.*	*millivolts*	*millivolts*		
1	2.030	59.6	1.845	0.185	558.2	236.6	5.213	6.212
2	69.38	59.1	1.760	67.62	707.5	236.6	7.633	6.048
3	3.813	59.7	1.891	1.922	616.2	236.8	6.150	6.143
4	2.291	62.0	1.843	0.448	568.7	236.8	5.480	5.995
5	2.216	58.3	1.715	0.501	576.5	237.1	5.502	6.036
6	13.40	59.5	1.843	11.56	660.5	236.8	6.868	6.070
7	1.993	58.2	1.816	0.177	554.1	237.1	5.139	6.150
8	1.970	58.1	1.792	0.178	551.8	236.7	5.108	6.110
9	13.33	59.9	1.880	11.45	660.9	236.7	6.876	6.091
10	2.096	61.4	1.870	0.226	559.9	236.6	5.241	6.159
11	3.387	61.7	1.940	1.447	608.2	236.8	6.020	6.147
12	16.63	58.6	1.801	14.83	668.4	236.7	6.998	6.082
13	3.105	58.4	1.831	1.374	595.3	236.5	5.816	5.973
14	2.189	58.2	1.819	0.370	573.0	236.6	5.453	6.144
15	15.51	58.9	1.835	13.67	667.3	236.7	6.980	6.108
16	4.897	58.5	1.791	3.106	624.7	236.4	6.294	6.055
17	1.990	56.2	1.761	0.229	589.8	236.3	5.244	6.130
18	2.115	59.4	1.833	0.282	564.1	236.8	5.306	6.119
19	2.031	58.6	1.815	0.216	556.3	236.8	5.179	6.103
20	8.38	60.2	1.885	6.49	648.2	236.7	6.670	6.133
21	2.371	60.3	1.827	0.544	575.8	236.4	5.501	6.027
22	2.333	60.3	1.912	0.421	577.2	236.4	5.524	6.182
Average..								6.101

* Individual values of α and total base for each urine are given in Table I.

Fig. 3 shows that the variations of pK′ in urine are related to variations in total base content. Hastings and Sendroy (1925) have shown that pK'_1 of carbonic acid, in NaCl-$NaHCO_3$ solutions containing mainly NaCl, can be expressed by the equation

$$pK' = 6.32 - 0.5\sqrt{\mu} \tag{5}$$

where μ is the ionic strength, 6.32 is the pK′ (= pK) at infinite dilution of the salts,[1] and the term $0.5\sqrt{\mu}$ expresses the depression of pK′ caused by salts. With univalent salts, like NaCl and $NaHCO_3$, the ionic strength is the same as the total base concentration in equivalents per liter. With polyvalent salts, the ionic strength is greater, being three times as great in the case of Na_2SO_4, or of $CaCl_2$. Since the urine electrolytes are in part polyvalent, it could be anticipated that, per unit total base concentration, the pK′ would be decreased somewhat more than in a NaCl-$NaHCO_3$ solution. Fig. 3 shows in fact that the equivalent depression in urine is somewhat greater, and that the relationship of pK′ to total base concentration is expressed by the equation

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[1] Correction by use of the later CO_2 factors of Van Slyke and Sendroy (1927) and the α values of Van Slyke, Sendroy, Hastings, and Neill (1928) affects the results of Hastings and Sendroy (1925) just enough to change their pK from 6.33 to 6.32.

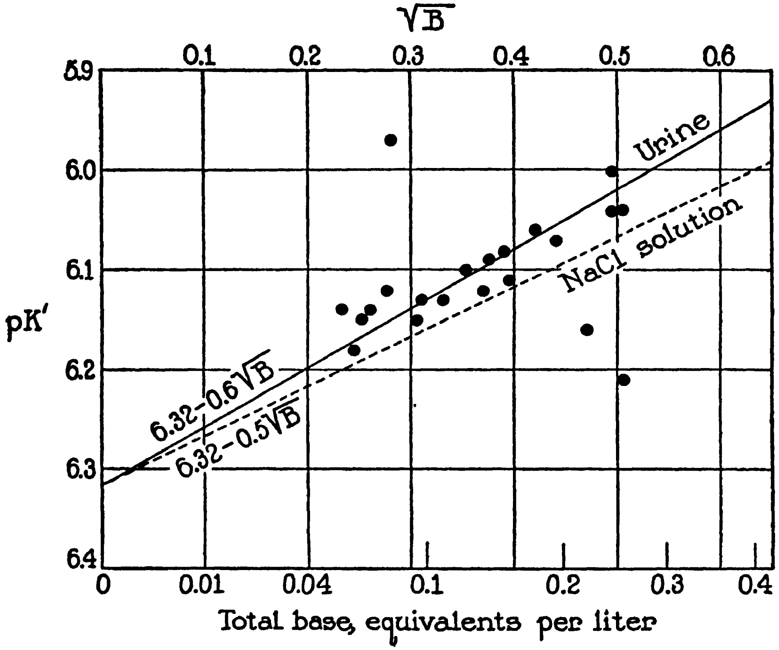

FIG. 3. The variation of urine pK′ with electrolyte (total base) concentration. All points and solid line are for urine. Dotted line for NaCl is taken from Hastings and Sendroy (1925).

$$pK' = 6.32 - 0.6\sqrt{B} \qquad (6)$$

where B represents the total base content in equivalents per liter. In eighteen of the twenty-two determinations Equation 6 expresses the value of pK′ within ±0.03 unit of the observed value.

Mainzer and Bruhn (1931) obtained pK′ values varying from 5.81 to 6.30, and averaging 6.13. They were unable to find any correlation between pK′ variation and freezing point lowering. Their average is near ours, and it is possible that in extremely dilute urines pK′ may approach as close to the pK for infinite dilution as their maximum value of 6.30. It is difficult to conceive, however, factors which could lower pK′ to Mainzer and Bruhn's minimum value. Total base rarely exceeds 0.4 equivalent per liter, which would correspond, by Equation 6, to a pK′ of 5.94. The technical error in determining pK′ is likely to be much greater in urines than in serum, or solutions of bicarbonate content similar to that of serum. The $BHCO_3$ in the urine is often only a fraction of a millimole per liter, and may be as little as 0.1 of the H_2CO_3. Consequently slight errors in calculating the H_2CO_3 from the CO_2 partial tension and the α used for the individual urine, would be multiplied in some cases as much as 10-fold in their effect on $BHCO_3$ calculated by difference. Of our own three pK′ values (Urines 1, 10, 13) which deviate markedly from Equation 6, two are from experiments in which the $BHCO_3$ concentration was only 0.3 mM or less. Thus, the deviations may in part be the result of the wider margin of experimental error that is unavoidable when $BHCO_3$ is so low. It appears possible that the wider fluctuations of Mainzer and Bruhn may have been caused by a somewhat wider margin of error in some of their determinations. A low value of α used in Equation 3 would cause a low value of pK′, and we have pointed out above that some of their α values appear to be improbably low.

Effect of Urea on pK′—Table III contains data indicating the effect which urea in 3 per cent (0.5 M) concentration has on the value of pK′ in bicarbonate-salt solution. The procedure followed for these experiments was exactly the same as that of Hastings and Sendroy (1925). The calculations were made according to Equations 1 and 2. The value of α used was calculated for each solution according to its composition, from the data of Van Slyke, Sendroy, Hastings, and Neill (1928) for the salt effect, and according to Usher (1910) for the urea effect. A urea concentration of 0.5 M was used because this approximates the maximum urea content of human urine. Urea in this concentration causes an average decrease in pK′ of only 0.013 unit. Variations in pK′ of urine

TABLE III

Effect of Urea on pK′ of Salt Solution at 38°

Sample No.	Initial composition				CO_2 tension	α	$[H_2CO_3]$	$[CO_2]$	$[NaHCO_3]$	pH	pK′	Average pK′	Δ pK′ per 500 mM urea per liter
	mM per l.	mM per l.	mM per l.	mM per l.	mm.		mM per l.	mM per l.	mM per l.				
1	$NaHCO_3$, 28.6				97.5	0.542	3.123	31.97	28.85	7.194	6.228	6.219	−0.003
1-a	" 28.6				95.4	0.542	3.057	31.93	28.87	7.185	6.209		
2	" 28.6	Urea, 500			97.6	0.534	3.083	31.83	28.75	7.176	6.206	6.216	
2-a	" 28.6	" 500			96.7	0.534	3.092	31.90	28.81	7.196	6.226		
3	$NaHCO_3$, 28.6		NaCl, 128.0		96.6	0.528	3.016	31.85	28.83	7.099	6.119		−0.019
4	" 28.6	Urea, 500	" 128.0		96.1	0.520	2.960	31.65	28.69	7.099	6.112	6.100	
4-a	" 28.6	" 500	" 128.0		97.2	0.520	2.989	31.82	28.83	7.073	6.088		
5	Na_2CO_3, 15.0		Na_2HPO_4, 24.0	KH_2PO_4, 6.0	48.7	0.534	1.538	30.06	28.52	7.428	6.159		−0.017
6	" 15.0	Urea, 500	" 24.0	" 6.0	30.4	0.526	0.946	27.90	26.95	7.596	6.142		
7	$NaHCO_3$, 30.0		Na_2HPO_4, 21.6	KH_2PO_4, 8.4	87.6	0.534	2.765	33.48	30.71	7.196	6.150		−0.011
8	" 30.0	Urea, 500	" 21.6	" 8.4	85.7	0.526	2.665	33.45	30.78	7.202	6.139		
Average													−0.013

exceeding 0.01 can therefore not be ascribed to variations in the urea concentration.

TABLE IV

Effect of Variation in α and pK′ on Accuracy of Henderson-Hasselbalch Equation Applied to Urine

Urine No.	CO_2 content	CO_2 tension	H_2CO_3 calculated		$BHCO_3$ calculated		pH calculated from (*a*) and (*b*) with		Error in pH calculated according to (*h*)
			From individual α (Table I) and (*b*)	From average α of 0.522, and (*b*)	From (*a*) − (*c*)	From (*a*) − (*d*)	Individual α and pK′ (Tables I and II)	Average α and pK′ of 0.522 and 6.10	(*g*) − (*h*)
	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)	(*g*)	(*h*)	(*i*)
	mM per l.	*mm.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*			
1	1.928	56.8	1.758	1.752	0.170	0.176	5.20	5.10	−0.10
2	75.37	119.0	3.542	3.671	71.83	71.70	7.36	7.39	+0.03
3	3.360	41.6	1.318	1.283	2.042	2.077	6.33	6.31	−0.02
4	2.115	59.4	1.764	1.832	0.351	0.283	5.29	5.29	0.00
5	2.481	68.8	2.023	2.122	0.458	0.359	5.39	5.33	−0.06
6	16.90	124.3	3.848	3.835	13.05	13.06	6.60	6.63	+0.03
7	1.898	52.6	1.641	1.623	0.257	0.275	5.35	5.33	−0.02
8	1.441	41.5	1.282	1.280	0.159	0.161	5.20	5.20	0.00
9	18.20	124.5	3.906	3.841	14.29	14.36	6.65	6.67	+0.02
10	2.595	69.8	2.123	2.153	0.472	0.442	5.51	5.41	−0.10
11	3.016	54.6	1.716	1.684	1.300	1.332	6.04	6.00	−0.04
12	18.28	73.0	2.242	2.252	16.04	16.03	6.94	6.95	+0.01
13	2.810	51.1	1.599	1.576	1.211	1.234	6.09	5.99	−0.10
14	2.127	56.9	1.778	1.755	0.349	0.372	5.43	5.43	0.00
15	16.22	76.8	2.391	2 369	13.83	13.85	6.87	6.87	0.00
16	6.009	84.0	2.571	2.591	3.438	3.418	6.18	6.22	+0.04
17	2.110	51.0	1.597	1.573	0.513	0.537	5.64	5.63	−0.01
18	2.621	74.6	2.301	2.301	0.320	0.320	5.26	5.24	−0.02
20	9.75	86.2	2.698	2.659	7.05	7.09	6.54	6.53	−0.01
21	2.724	69.6	2.108	2.147	0.616	0.577	5.49	5.53	+0.04
22	2.188	59.1	1.857	1.823	0.33[1]	0.365	5.43	5.40	−0.03
Average..........									±0.032

Accuracy of Henderson-Hasselbalch Equation Applied to Urine

Table IV indicates the accuracy with which the *average* α and pK′ values may be applied to urine acid-base balance calculations by Equation 3. The pH values in column (*h*) have been calculated by the use of average α and pK′; those in column (*g*) have been derived by the use of the individually determined α and pK′ values of the same urines, as given in Tables I and II. Evidently urine pH may be calculated from accurately determined CO_2 content and CO_2 tension values, with an exactness at least equalling that of the colorimetric method (Hastings, Sendroy, and Robson, 1925).

Construction of Nomogram of Fig. 4

For calculation of one of the three variables, CO_2 content, CO_2 tension, or pH from the other two in urine, a line chart for graphic solution of the Henderson-Hasselbalch equation is convenient. The nomogram in Fig. 4 accurately presents total CO_2 as a linear function of pH and CO_2 tension. It is theoretically exact, and is in this respect an improvement over the blood plasma line chart of Van Slyke and Sendroy (1928, p. 783). A straight line drawn through given points on two of the scales will intercept the third scale at a point corresponding to the relationship expressed by Equation 3, with $\alpha = 0.522$ and $pK' = 6.10$. By the use of appropriate α and pK′ values, a similar chart could be constructed for blood plasma calculations.

If the fraction of the total CO_2 in the form of the free acid, symbolized for convenience as H_2CO_3, be denoted by f, we may write

$$(7) \qquad f = [H_2CO_3]/[CO_2], \text{ and } \log f = \log [H_2CO_3] - \log [CO_2]$$

We have then, a straight line logarithmic relationship in the form of $\log x = \log y + \log z$. If Equation 2 be written in the form

$$(8) \qquad pH = pK' + \log \frac{1-f}{f}$$

then it is obvious that we have another logarithmic expression for f; namely,

$$(9) \qquad \log f = pK' - pH + \log (1 - f)$$

Since given values of $[CO_2]$ and $[H_2CO_3]$ (or CO_2 tension) fix the value of f they also fix the value of pH.

For Fig. 4, the values of $\log [CO_2]$ and $\log [H_2CO_3]$ in terms of millimoles per liter, were spaced off on parallel axes of equal length

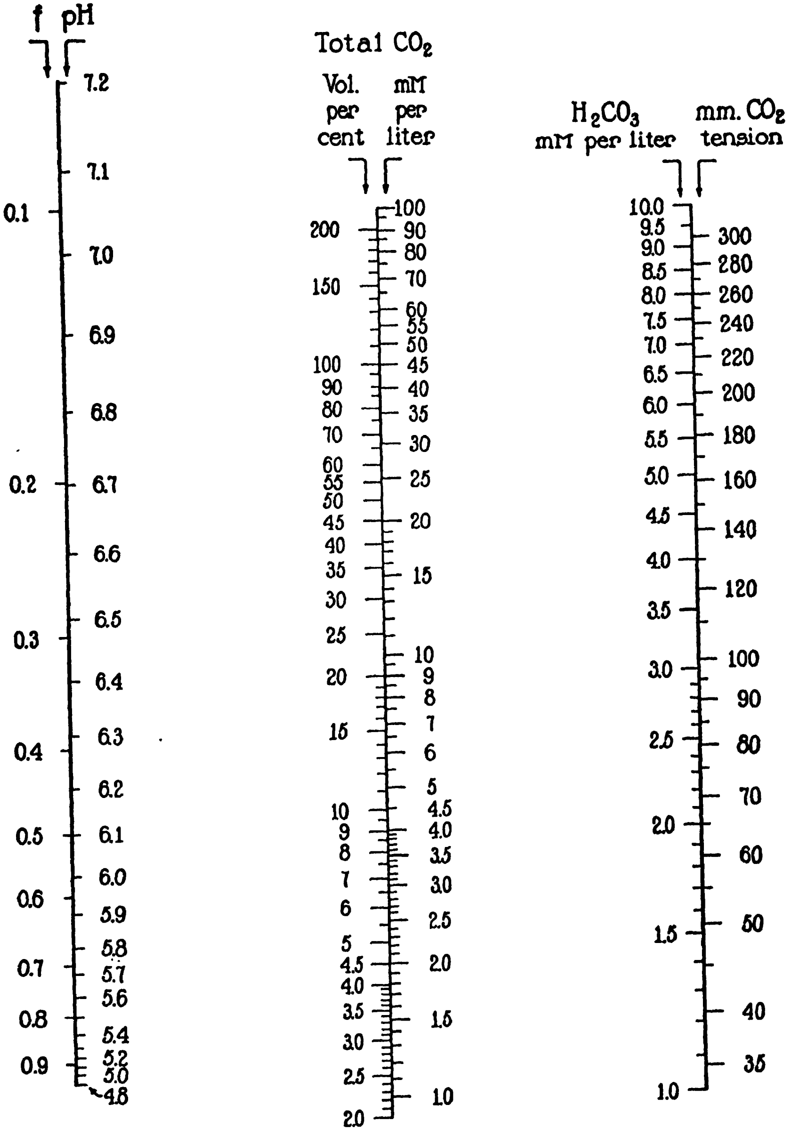

Fig. 4. Nomogram for calculation of acid-base balance of urine. A straight line drawn between values on any two scales intersects the third at a value corresponding to the relationship expressed by the Henderson-Hasselbalch equation.

drawn on rectangular coordinate paper. An equal distance away, another parallel axis of equal length was spaced off for values of log f. By Equation 8 values of f corresponding to given pH values were calculated, and located logarithmically on the f axis. The points found were marked with values of the given corresponding pH. Points corresponding to CO_2 tension values were marked off on the H_2CO_3 scale. For these calculations the average values $\alpha = 0.532$ and $pK' = 6.10$ were used.

SUMMARY

The solubility coefficient of CO_2 in twenty-two human urines was found to average 0.522 ± 0.08 at 38°. The maximum range of variation was from 0.498 to 536. The variation was chiefly a function of the salt concentration.

The value of Hasselbalch's constant, pK', in human urine was found to average 6.10 ± 0.07. The maximum range of variation was from 5.97 to 6.21. This variation was also found to be chiefly a function of the salt concentration, according to the approximate equation, $pK' = 6.32 - 0.6\sqrt{B}$, where B represents equivalents of total base per liter of urine. The value of pK', in solutions of the ionic strengths encountered in urine, was found to be changed by only 0.01 by addition of urea in the highest concentration (0.5 M) found in human urine.

The use of average α and pK' values in calculation of urine pH from CO_2 tension and CO_2 content yielded pH values with an average error of ±0.03 and a maximum error of 0.10.

A line chart for urine has been constructed, with scales for CO_2 content, CO_2 tension, and pH, whereby any one of these variables can be calculated from known values of the other two.

BIBLIOGRAPHY

Fiske, C. H., *J. Biol. Chem.*, **51**, 55 (1922).

Gamble, J. L., *J. Biol. Chem.*, **51**, 295 (1922).

Hasselbalch, K. A., *Biochem. Z.*, **78**, 112 (1916).

Hastings, A. B., and Sendroy, J., Jr., *J. Biol. Chem.*, **65**, 445 (1925).

Hastings, A. B., Sendroy, J., Jr., and Robson, W., *J. Biol. Chem.*, **65**, 381 (1925).

Hastings, A. B., Sendroy, J., Jr., and Van Slyke, D. D., *J. Biol. Chem.*, **79**, 183 (1928).

Henderson, L. J., *J. Biol. Chem.*, **7**, 29 (1909-10).

Hoffman, W. S., *J. Biol. Chem.*, **93**, 787 (1931).
Mainzer, F., and Bruhn, M., *Biochem. Z.*, **230**, 395 (1931).
Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Baltimore, **2** (1932).
Sendroy, J., Jr., Seelig, S., and Van Slyke, D. D., *Sunti communicaz. scient., XIV cong. internaz. fisiol.*, Rome, 232 (1932).
Usher, F. L., *J. Chem. Soc.*, **97**, 66 (1910).
Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, **61**, 523 (1924).
Van Slyke, D. D., and Sendroy, J., Jr., *J. Biol. Chem.*, **73**, 127 (1927); **79**, 781 (1928).
Van Slyke, D. D., Sendroy, J., Jr., Hastings, A. B., and Neill, J. M., *J. Biol. Chem.*, **78**, 765 (1928).
Van Slyke, D. D., Sendroy, J., Jr., and Liu, S. H., *J. Biol. Chem.*, **95**, 531 (1932).

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STUDIES OF ACIDOSIS

XXIII. THE CARBON DIOXIDE TENSION AND ACID-BASE BALANCE OF HUMAN URINE*

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(Received for publication, June 15, 1934)

Marshall (1922) and Gamble (1922) were the first to correlate CO_2 content and pH in urine. They agreed in finding that the CO_2 content increased with the pH, urines of high alkalinity showing a bicarbonate content up to several grams per liter. Gamble further calculated the free carbonic acid, customarily symbolized as H_2CO_3, from observed pH and CO_2 content of the urine by means of the Henderson-Hasselbalch equation. He assumed for pK′ a value of 6.10, which had previously been found for blood plasma, and which in fact we now find to be the average value for urine also (Sendroy, Seelig, and Van Slyke, 1934). Urine CO_2 tension values calculated from Gamble's H_2CO_3 results, with Sendroy, Seelig, and Van Slyke's average value of 0.522 for α, indicate a variation from 51 to 76 mm., with an average of 61 mm.

Mainzer, with his collaborators (1929, 1931), has since studied the problem further. Like Gamble and Marshall, he measured directly, in the freshly collected urine, only the pH and total CO_2 content, and calculated the CO_2 tension from these values by means of the Henderson-Hasselbalch equation. In the second paper, Mainzer and Bruhn (1931) measured the pH with the quinhydrone electrode, and for each sample of urine they made separate determinations of pK′ and α for use in the calculation. The extent of the variations in pK′ and α encountered in different urine samples has already been indicated in the preceding paper (Sendroy, Seelig, and Van Slyke, 1934), in which we have compared Mainzer and Bruhn's results with our own. Mainzer and Bruhn's calculated CO_2 tensions in freshly voided urine varied between 13 and 242 mm., over an immensely wider range than those of Gamble. Mainzer concluded that there was no relationship between CO_2 tension in urine and CO_2 tension in blood.

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* A preliminary communication concerning this work was delivered at the XIVth International Physiological Congress at Rome (Sendroy, Seelig, and Van Slyke, 1932).

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The wide discrepancy between the urinary CO_2 tension ranges reported by Mainzer and those calculable from Gamble's data made a reexamination of the problem desirable. These authors did not make direct measurements of the CO_2 tension, but calculated it by the Henderson-Hasselbalch equation from the pH and total CO_2 content. In such calculations the combined errors in pH and CO_2 content determinations, and in the values for pK′ and α used in the Hasselbalch equation, all accumulate on the CO_2 tension.

In order to avoid such cumulative errors we have returned to direct measurement of the CO_2 tension by the tonometric procedure, introduced by Pflüger (1872) for estimating the gas tensions in blood, and applied to urine by Strassburg (1872). A relatively large volume of urine is equilibrated with a relatively small volume of gas until the latter attains the CO_2 tension of the urine. The tension is then ascertained by analysis of the gas.

Van Slyke, Sendroy, and Liu (1932) have applied this principle to the direct measurement of p_{CO_2} in blood,[1] and the technique used for urine is an adaptation of the same procedure. Since most samples of urine are low in CO_2 concentration, and many may be also low in buffer value, and because the extent of p_{CO_2} variation in urine is much greater than in blood, the ratio of gas volume to liquid volume has been changed from 1:9 for blood to 1:50 for urine. It has also been found necessary to prolong the equilibration time to ½ hour.

PROCEDURE

Method for Direct Measurement of Urine CO_2 Tension

Preparation of Gas Mixture—It is desirable that the gas mixture used have a CO_2 tension within 25 mm. of the urinary p_{CO_2}. One can calculate that if 50 volumes of buffer-free water, containing CO_2, only in the form of free carbonic acid, are equilibrated with 1 volume of gas, 25 mm. difference between gas and water with respect to initial p_{CO_2} will produce in the equilibrated system a final p_{CO_2} which differs by 1 mm. from the initial p_{CO_2} of the water. The gas tension will move 24 mm. towards the water tension, and the latter will move 1 mm. towards the initial gas tension. If the water contains buffers active at its pH, the final p_{CO_2} will be less than 1 mm. from the initial p_{CO_2} of the solution. Urine does contain such buffers, notably bicarbonate and phosphate, so that the correction to the final p_{CO_2} for initial difference in p_{CO_2} between urine and gas bubble is less than one-twenty-fifth of that difference. However, the buffer content of urine varies so greatly that it is impossible to estimate a routine correction for initial p_{CO_2} differences between urine and the gas bubble. One can only state that the error from failure to make such correction will be less than 1 mm. if the initial difference in p_{CO_2} between urine and gas bubble does not exceed 25 mm.

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[1] The symbol p_{CO_2} is used to indicate CO_2 tension in mm. of mercury.

Accordingly, in our procedure the urine is equilibrated with an air-CO_2 mixture having a p_{CO_2} of 67 mm., the average p_{CO_2} of urine. This is within 25 mm. of the p_{CO_2} of the great majority of urines. If the final p_{CO_2} found by analysis of the equilibrated bubble is outside the range 67 ± 25 mm., a second portion of urine is equilibrated with gas that has a CO_2 tension approximating that found for the urine by the preliminary estimation. Since urinary p_{CO_2} is practically never below 40 mm., a second equilibration will be necessary only for urines with unusually high tensions. In such cases the urine is likely to be highly buffered, so that the error in the preliminary determination is usually not significant; but in order to make certain of accuracy, a second equilibration with gas containing more CO_2 is desirable.

For routine use, it is convenient to have on hand large amounts of the air-CO_2 mixture with 67 mm. p_{CO_2}. The percentage of CO_2 in such a gas mixture is calculated as $67/(0.01\ (B - 50))$, where B is the average barometric pressure. At sea level the CO_2 content of the desired mixture is 9.5 per cent. Our gas mixtures were prepared and stored in 300 cc. containers as described by Van Slyke, Sendroy, and Liu (1932, p. 554).

The other CO_2-air mixtures occasionally required for urines with higher CO_2 tensions may be prepared by mixing measured volumes of air and CO_2 in a gas burette. In our experiments, however, we prepared such mixtures by attaching the tonometer, before the urine was placed in it, directly to the gas manifold. The system was partially exhausted and the required pressure of CO_2 was added, measured by a manometer, as described on p. 302 of Peters and Van Slyke (1932).

Tonometer—The tonometer in which the equilibration takes place resembles that described by Van Slyke, Sendroy, and Liu, except that the body has a capacity of 50 cc. instead of 9 cc. for the liquid phase. The gas bubble volume is 1 cc.

Equilibration of Urine and Gas at 38°—The tonometer is first filled with the desired gas mixture. Enough urine (>100 cc.) for duplicate p_{CO_2} determinations is voided under oil, and transferred at once to a container over mercury, from which it is introduced into the tonometer to fill all but 1 cc. of its volume. Urine and gas are equilibrated at 38° as described by Van Slyke, Sendroy, and Liu (1932) for blood samples, except that the length of time required for complete equilibration is extended to ½ hour. The vessel is opened to the air after 5, 10, and 15 minute periods to keep the pressure at atmospheric, and is then rotated for another 15 minutes. Satisfactory results have been obtained with some samples of urine rotated for a shorter time, but for uniformly good results, for widely different samples of urine, 30 minutes have been found necessary.

Analysis of Gas Bubble—This is carried out as described by Van Slyke, Sendroy, and Liu (1932). The gas sample measurement by manometric pressure is made with the gas at 2.0 cc. volume. The pressure of the CO_2 gas is usually measured at 0.5 cc. volume. However, some samples of urine high in CO_2 tension yield so much CO_2 to the bubble, that measurement of the CO_2 pressure must be made with the extracted CO_2 at 2.0 cc. volume.

Calculation

The CO_2 tension is calculated from the volume per cent CO_2 content, C, of the gas bubble, according to the usual equation

$$p_{CO_2} = 0.01\,C\,(B - 49.7) \tag{1}$$

where B is the barometric pressure in mm. of mercury at the time of the gas bubble equilibration, and 49.7 is the vapor tension of water at 38°.

EXPERIMENTAL

Comparison of Known Urine CO_2 Tensions with Tensions Found by Present Method

Urine samples of about 110 cc. were saturated at 38° with known mixtures of CO_2 and air, in a two-chambered saturating vessel by the "First saturation method" of Austin *et al.* (1922). After complete gaseous equilibrium had been attained, the gas and liquid phases were separated, and the gas was analyzed for CO_2 in a Haldane apparatus. From the CO_2 content of the gas, the CO_2 tension of it, and therefore of the urine, was calculated with an error not exceeding 0.2 mm. The tensions thus obtained are given in Table I as "CO_2 tension of urine at 38°, present." The urine was then used for duplicate determinations of the CO_2 tension by the present method.

The results, recorded in Table I, indicate that the error of urine CO_2 tensions measured as described in this paper averaged 1.1 mm. The maximum error found, occurring in but one case, was 3.1 mm.

In this series of experiments, the maximum difference between the initial p_{CO_2} of the bubble and the true p_{CO_2} of the urine was 31 mm. It does not appear from the results that initial differences within this limit have a significant effect on the final p_{CO_2} determined.

CO_2 Tension of Freshly Voided Human Urine

60 individual samples of urine were analyzed by the procedure for direct p_{CO_2} presented in this paper. The urines were taken at intervals varying from 1 to 10 hours after previous voiding, and under various conditions of activity. Each sample was collected at a single voiding.

For the first analysis in each case the initial tension of the bubble was set at 67 mm. The second analysis was performed with p_{CO_2} of the bubble approximating the value obtained by the first analysis. When the urine p_{CO_2} was over 100 mm. the result of the second analysis was taken as the p_{CO_2} value. Except when values above 130 mm. were encountered, the agreement between the two analyses was within the limit of error of the method.

Other data recorded for each specimen were specific gravity, pH, and the period of urine retention for the particular voiding analyzed. The specific gravity measurements were made with an ordinary urine specific gravity bulb; their maximum error is about 0.003. The pH values were obtained colorimetrically according to the method of Hastings, Sendroy, and Robson (1925) but with the use of a Hastings-Duboscq colorimeter in place of the acid-alkali standard color tubes (Peters and Van Slyke, 1932, p. 801).

TABLE I

Urine CO_2 Tensions Found by Present Method Compared with Tensions Set by Saturation with Known CO_2-Air Mixtures

Urine No.	Initial CO_2 tension of gas bubble at 38°	CO_2 tension of urine at 38°		
		Present	Found from final p_{CO_2} of gas bubble	Error
	mm.	*mm.*	*mm.*	*mm.*
1	82	90.6	89.2	−1.4
2	82	98.2	99.6	+1.4
3	82	95.9	94.4	−1.5
4	82	104.9	105.3	+0.4
5	82	98.6	97.1	−1.5
6	82	99.9	97.8	−2.1
7	82	96.0	96.5	+0.5
8	82	97.8	97.3	−0.5
9	82	99.6	96.5	−3.1
10	82	99.3	98.9	−0.4
11	82	99.2	99.1	−0.1
12	82	67.9	69.1	+1.2
13	79	62.7	61.0	−1.7
14	82	55.7	56.2	+0.5
15	58	32.7	34.3	+1.6
16	43	23.4	23.4	±0.0
17	172	140.7	139.7	−1.0
18	172	159.5	158.6	−0.9
19	96	80.1	81.1	+1.0
20	96	75.4	76.5	+1.1
Average				±1.1

The urine specimens examined fell into three groups: normal, pathological, and experimental. The first group consisted of thirty specimens obtained from normal individuals under ordinary conditions. The second group consisted of fourteen specimens obtained from patients at this hospital suffering from nephritis or cardiac disease. The third group of sixteen specimens was obtained from normal individuals whose acid-base balance was purposely shifted from normal by the administration of alkali or by exercise.

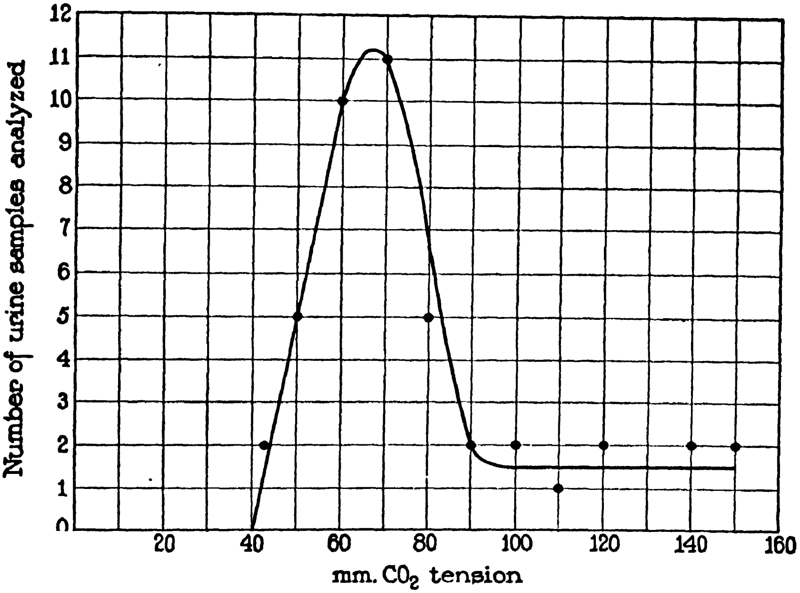

FIG. 1. Distribution curve of normal and pathological human urine CO_2 tension results at 38°.

RESULTS

In Fig. 1 is given the distribution curve for urine p_{CO_2} results found in our study of forty-four normal and pathological cases. The urine p_{CO_2} of the normal individuals varied from 53 to 149 mm.

The variation in pathological cases covered nearly the same range, from 42 to 124 mm.

Fig. 1 indicates that in our study there are more naturally occurring urine CO_2 tensions at 67 ± 5 mm. than at any other level, 25 per cent of the results being at that level. Furthermore, 77 per cent of the results are between 42 and 95 mm. The curve is peculiar in that there is no asymptotic approach to the abscissa on either end. The lower end of the curve cuts the abscissa at a sharp angle, since no results were found for urine p_{CO_2} below 40 mm. The upper end of the curve runs from 95 mm. to 149 mm. parallel to the abscissa.

TABLE II

CO_2 Tension in Urine after Experimental Alteration of Individual Acid-Base Balance

Urine No.	Specific gravity	Period	CO_2 tension of urine by bubble analysis	pH	Treatment
		hrs.	*mm.*		
1	1.024	10¼	119	7.4	23 gm. $NaHCO_3$
2	1.008	2	42	6.3	H_2O drinking
3	1.018	3	124	6.5	5 gm. Na citrate
4	1.023	3¼	169	5.8	Exercise, playing squash
5	1.025	3¼	159		" stair-running
6	1.032	3¾	136	7.2	5 gm. $NaHCO_3$ + stair-running
7	1.015	4½	58	5.3	Stair-running
8	1.026	1¾	183	5.1	Exercise, stair-running
9	1.026	2½	70	5.5	5 gm. $NaHCO_3$ + stair-running
10	1.009	2	55	6.0	Exercise, stair-running
11	1.010	2	73	5.5	5 gm. $NaHCO_3$ + stair-running
12	1.007	2¼	51	6.1	Exercise, stair-running
13	1.019	3	57	5.4	HCl + stair-running
14	1.032	3	52	5.0	Exercise
15	1.035	2½	201	7.5	5 gm. $NaHCO_3$ + stair-running
16	1.024	2¼	177	4.9	Exercise, stair-running

When the sixteen experimental cases recorded in Table II are also considered, there is found little to alter this picture. The lower limit is 42 mm. for urine p_{CO_2} obtained after dilution by drinking large amounts of water (Urine 2). The upper limit is 201 mm. obtained after the ingestion of 5 grams of $NaHCO_3$ and severe exercise (stair-running, 30 meters up and down, three times, Urine 15). Indeed, *all of the five results higher than 150 mm.*

among our total number of 60 urine sample determinations *were obtained after the subject had exercised.* Results between 100 and 150 mm. were usually obtained after the ingestion of alkali in the form of citrate or bicarbonate. A few high results were also obtained in apparently normal subjects under ordinary conditions.

Relationship between Urine CO_2 Tension and Specific Gravity

Fig. 2 indicates that none of our urine samples with specific gravity lower than 1.018 was of a CO_2 tension higher than 80 mm. Higher CO_2 tensions occurred only in fairly concentrated urines.

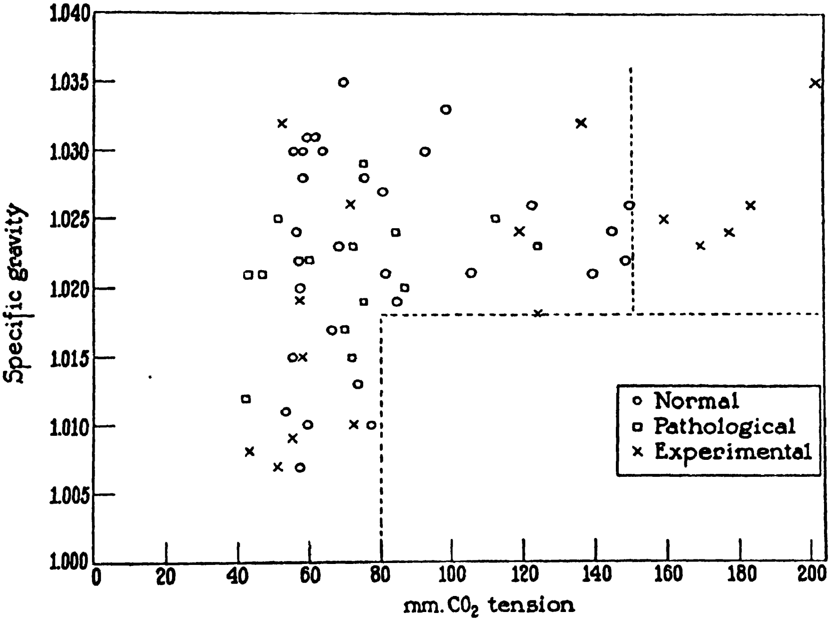

FIG. 2. The relation between CO_2 tension of urine and the specific gravity

Relationship between Urine CO_2 Tension and Period of Accumulation in the Bladder

Fig. 3 shows that no urine retained over 4 hours had a CO_2 tension over 120 mm., while a third of those retained less than 4 hours had tensions over 120 mm., ranging up to 200 mm. Apparently, high CO_2 tensions are to be found only in concentrated urines stored in the bladder for a short period, but the converse of this is not true; namely, that high concentration or short period of accumulation, or both, result uniformly in high CO_2 tensions.

A possible explanation for the absence of high CO_2 tensions (greater than 120 mm.) in urine retained over 4 hours is that prolonged accumulation in itself tends to produce a urine of average composition. If temporary conditions produce a urine of high CO_2 tension during 1 hour, it may be diluted with urine of usual tension during subsequent hours. In the one observation providing an exception to the 4 hour rule (Urine 1, Table II), an abnor-

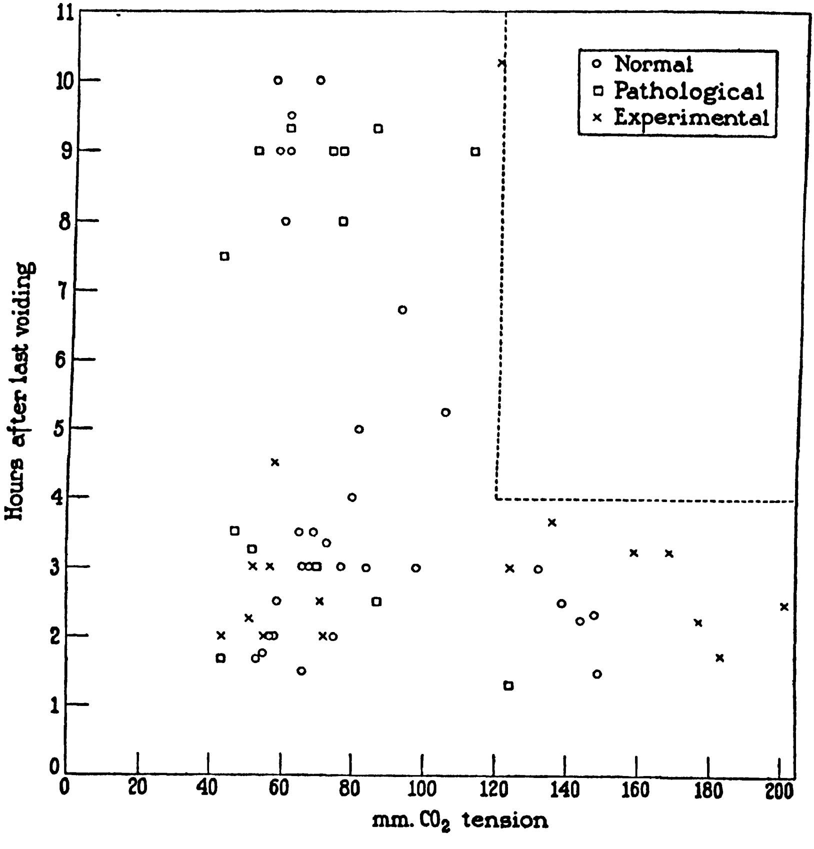

FIG. 3. The relation between CO_2 tension of urine and the period of accumulation in the bladder.

mal state was prolonged by taking 15 grams of $NaHCO_3$ in the evening and 8 grams more in the morning before voiding. The urine, voided after 10 hours collection, had a high CO_2 tension.

An alternative explanation is that free carbonic acid, which is one of the most diffusible of physiological solutes, slowly diffuses through the bladder wall when the CO_2 tension of the urine is higher than that of the blood.

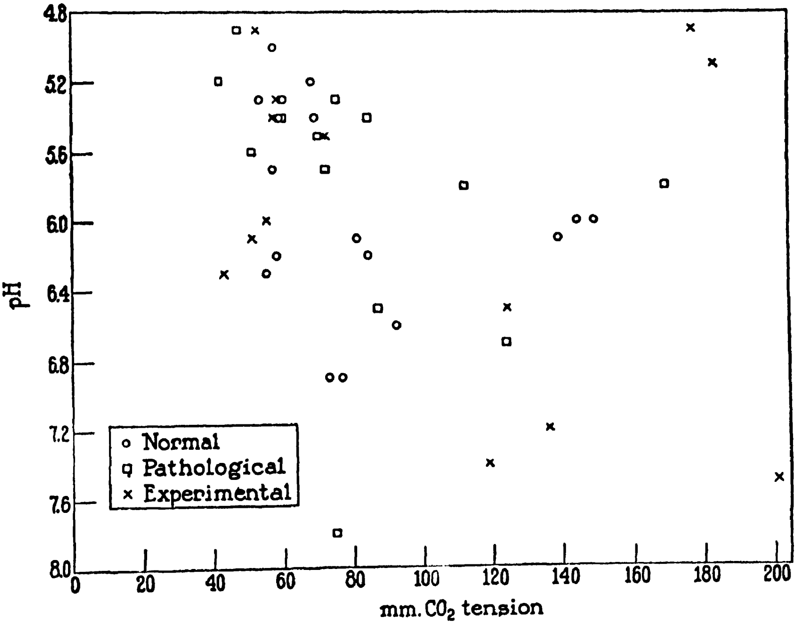

FIG. 4. The relation between CO_2 tension and pH of urine

In general, one may say that dilute urines, or urines stored in the bladder for a long period, tend to approach the average value of 67 mm. for CO_2 tension (see upper left corner of Fig. 3, and lower left corner of Fig. 2). Herein may lie the explanation for Gamble's (1922) comparatively constant urinary H_2CO_3 values.

Absence of Relationship between CO_2 Tension and pH

As is indicated by Fig. 4, we have found in agreement with Mainzer and Bruhn (1931), no correlation between the pH and the CO_2 tension of urine.

TABLE III

Summary of Urine CO_2 Tension Values Recorded in Literature

Author	Method	Urine samples		CO₂ tension			Remarks
		No. analyzed	Species	Variation	Mean	Distribution	
				mm.	*mm.*		
Strassburg (1872)............	Direct, tonometric	3	Dog	56– 84	70		Fresh samples
Fredericq (1910).............	Direct, tonometric	43	Human	48–137	77		" "
		6	Dog	50–100	84		
Gamble (1922)...............	Calculated from CO_2 and pH	55	Human	48– 76		80%, 55–75 mm.	" "
Mainzer and Shen (1929).....	By interpolation on CO_2 absorption curves	7	"	43–102	81	All but two higher than 86 mm.	" "
" " Bruhn (1931)...	Calculated from CO_2 and pH with individual α and pK′ values	20	"	13–242		Only four (13, 17, 35, and 39 mm.) below 40 mm.	" " all subjects with $NaHCO_3$ or H_2O administered
Present authors (1934).......	Direct, tonometric	30	" normal	53–149		25% at 67 mm., 80%, 42–95 mm.	
		14	" pathological	42–124			
		16	" experimental	43–201			

Urine CO_2 Tensions Found by Other Authors

In Table III we have summarized the results available in the literature. The data of Strassburg (1872), Fredericq (1910), and our own are the only ones obtained by direct tonometric methods. Fredericq's results for forty-three human urine p_{CO_2} values, from 48 to 137 mm., agree well with our forty-four values from 42 to 149 mm. The other data have been sufficiently discussed in the introduction.

Acid-Base Balance of Freshly Voided Human Urine

With the results of this and of the preceding paper (Sendroy, Seelig, and Van Slyke, 1934) we are able to estimate for twenty-one freshly voided human urines the acid-base balance, in the terms of pH, $[CO_2]$, and p_{CO_2} commonly used to define the acid-base balance of the blood. With the individual α and pK′ values found for each urine, the pH was calculated according to the Henderson-Hasselbalch equation

$$\text{(2)} \qquad \text{pH} = \text{pK}' + \log \frac{[CO_2] - 0.1316\,\alpha\,p_{CO_2}}{0.1316\,\alpha\,p_{CO_2}}$$

with CO_2 content in terms of volume per cent.

The results are contained in Table IV of this paper, and are plotted in Fig. 5 on the logarithmic chart used by Peters and Van Slyke ((1931) p. 915). Fig. 5 is convenient for charting acid-base data in fields showing normal and abnormal variations. For its construction, the average values of $\alpha = 0.522$ and $\text{pK}' = 6.10$ were used in Equation 2 above, or, more conveniently, in the transformed equation

$$\text{(3)} \qquad [CO_2] = 0.1316\,\alpha\,(10^{\text{pH}-\text{pK}'} + 1)\,p_{CO_2}$$

The magnitude of error introduced by using average α and pK′ values is indicated in the preceding paper (Sendroy, Seelig, and Van Slyke, 1934).

In addition to the results of Table IV, we have also plotted the positions, in Fig. 5, of other urine samples, for which we have determined direct p_{CO_2} and colorimetric pH values. Included in the same chart are the results of Marshall (1922) from CO_2 content and colorimetric pH values, those of Gamble (1922) from CO_2 content and calculated p_{CO_2}, and those of Mainzer and Bruhn (1931) likewise from CO_2 content and calculated p_{CO_2} values.

There is rather good agreement between our results and those of Mainzer and Bruhn, in that there is a similarly wide scattering of points outside of the area in which the majority of urines fall. This majority area may be considered as bounded by the limits

TABLE IV

Acid-Base Balance of Freshly Voided Urine at 38°

Urine No.	α_{CO_2} determined	pK′ determined	CO_2 tension determined	CO_2 content determined		pH calculated by Equation 2
	cc. CO_2 per cc. solution		*mm.*	*mM per l.*	*vol. per cent*	
1	0.524	6.21	56.8	1.928	4.292	5.20
2	0.504	6.05	119.0	75.37	167.77	7.36
3	0.536	6.14	41.6	3.360	7.479	6.33
4	0.503	6.00	59.4	2.115	4.708	5.29
5	0.498	6.04	68.8	2.481	5.523	5.39
6	0.524	6.07	124.3	16.90	37.62	6.60
7	0.528	6.15	52.6	1.898	4.225	5.35
8	0.523	6.11	41.5	1.441	3.208	5.20
9	0.531	6.09	124.5	18.20	40.51	6.65
10	0.515	6.16	69.8	2.595	5.776	5.51
11	0.532	6.15	54.6	3.016	6.714	6.04
12	0.520	6.08	73.0	18.28	40.69	6.94
13	0.530	5.97	51.1	2.810	6.255	6.09
14	0.529	6.14	56.9	2.127	4.735	5.43
15	0.527	6.11	76.8	16.22	36.11	6.87
16	0.518	6.06	84.0	6.01	13.28	6.18
17	0.530	6.13	51.0	2.110	4.687	5.64
18	0.522	6.12	74.6	2.621	5.834	5.26
20	0.530	6.13	86.2	9.75	21.70	6.54
21	0.513	6.03	69.6	2.724	6.064	5.49
22	0.532	6.18	59.1	2.188	4.870	5.43

pH 5.0 to 6.4, p_{CO_2} 40 to 85 mm., and CO_2 content 3 to 13 volumes per cent, or 1.3 to 6 mM per liter.

Compatibility of Results with Filtration-Reabsorption Theory of Urine Secretion

The recent work of Richards (quoted below) and of Smith and his collaborators (Jolliffe, Shannon, and Smith, 1932; Shannon,

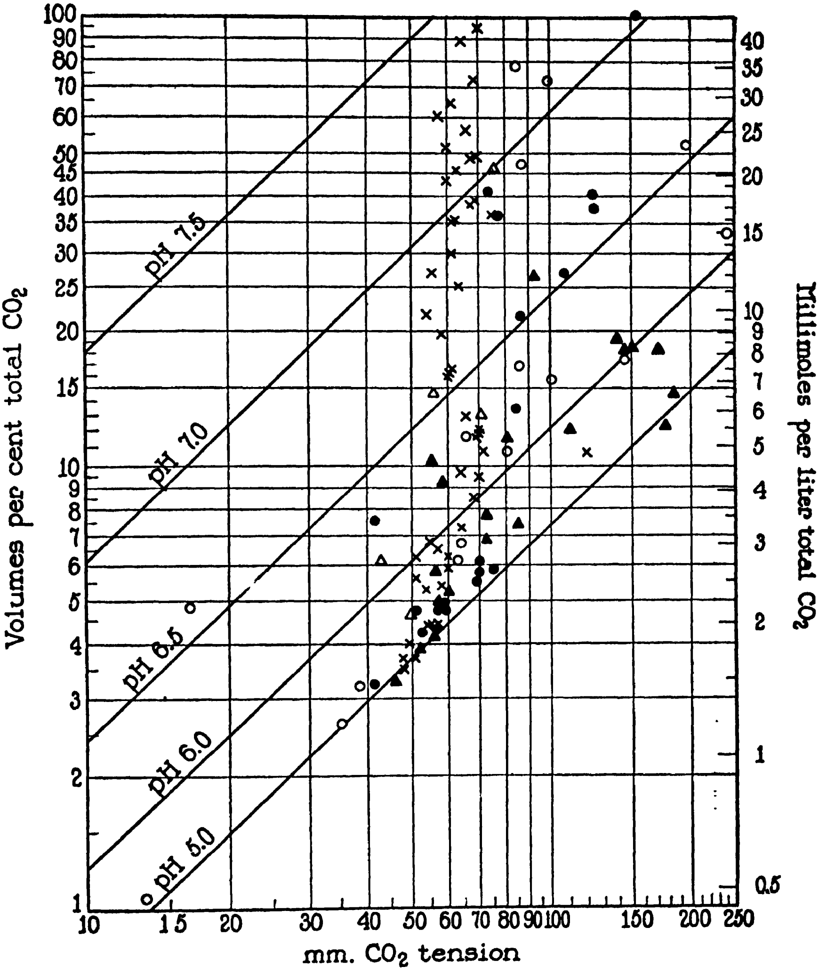

FIG. 5. Acid-base balance results for human urine. ○ Mainzer and Bruhn (1931), from CO_2 content and tension; × Gamble (1922), from CO_2 content and tension; △ Marshall (1922), from CO_2 content and pH; ● authors (1934), from CO_2 content and tension; ▲ authors (1934), from CO_2 content and pH.

Jolliffe, and Smith, 1932) has provided strong experimental support for this theory, and it appears of interest to ascertain whether the present results concerning the acid-base balance of the urine are in accord with it. As the simplest way in which to demonstrate such agreement we outline the sequence of events which, according to the theory, lead to the production of urine showing the demonstrated relationships of $BHCO_3$, H_2CO_3, and pH. The explanation outlined is tentative, but to avoid repeated reservations, the events are sketched as though the filtration-reabsorption mechanism were proved.

Formation of Acid Urine.—In the glomerular filtrate H_2CO_3 and $BHCO_3$ are presumably present in approximately the same concentrations as in the blood plasma. It is true that these concentrations of H_2CO_3 and $BHCO_3$ have not been analytically demonstrated in the filtrate, but Wearn and Richards (1924) have shown that the filtrate has the same pH as the plasma, so that the ratio, $H_2CO_3:BHCO_3$, is the same as in the plasma. Furthermore, when the other crystalloids, Cl, urea, uric acid, glucose, creatinine, PO_4, are filtered with unchanged concentrations, as shown by Richards and his collaborators, it appears reasonable to assume that H_2CO_3 and $BHCO_3$ are similarly filtered.

As the filtrate proceeds down the tubules it ordinarily becomes more acid, as has been colorimetrically demonstrated in the frog kidney by Richards (1929). It appears probable that the *acidification is the result of active reabsorption of* $BHCO_3$ in the same way that BCl is reabsorbed, the tubular cells returning both BCl and $BHCO_3$ from the filtrate to the blood as completely as may be necessary to maintain the normal supply of these salts in the body. In the cases of both chloride and bicarbonate, reabsorption may be so complete that only traces remain in the urine. In human urine, of the average normal pH of 6.1, the $BHCO_3$ concentration is only about one-tenth as great as in the blood plasma (Fig. 5). Table V illustrates, by average values, the estimated amounts of $BHCO_3$, H_2CO_3, and PO_4 which are passed into the glomerular filtrate, and the amounts which escape reabsorption and appear in the urine.

If one assumes that the glomerular filtrate forms in accordance with the filtration-reabsorption theory, and in the amounts indicated by either the xylose or the creatinine clearances (Jolliffe, Shannon, and Smith, 1932; Shannon, Jolliffe, and Smith, 1932), it appears difficult to explain the smallness of the amounts of $BHCO_3$ found in acid urine by any process other than tubular reäbsorption. Calculated as $NaHCO_3$, the amount of bicarbonate filtered in the 24 hour glomerular filtrate of about 150 liters would be about 340 grams, containing 92 grams of Na, while in the daily output of urine only 3 or 4 grams of Na are ordinarily found, chiefly in the form of chloride, and only a fraction of a gram of alkali bicarbonate (Table V).

TABLE V

Estimated 24 Hour Filtration and Excretion of $BHCO_3$, H_2CO_3, and PO_4 by Human Kidney

Substance	Glomerular filtrate,* 150 liters at pH = 7.4		Urine, 1.5 liters at pH = 6.1		Urine, 1.5 liters at pH = 5.0	
	Concentration	Total amount	Concentration	Total amount	Concentration	Total amount
	mM per l.	*mM*	*mM per l.*	*mM*	*mM per l.*	*mM*
$BHCO_3$	25	3700	2	3	0.1	0.15
H_2CO_3	1.3	195	2	3	1.3	2
PO_4	1	150	43	65	43	65

* From the results of Jolliffe, Shannon, and Smith (1932) it is assumed that the glomerular filtrate is about 1.4 times the maximum urea clearance. Møller, McIntosh, and Van Slyke (1928) have found the maximum urea clearance to average 75 cc. per minute in normal men. Hence the glomerular filtrate is calculated to be $1.4 \times 75 = 10.5$ cc. per minute, and $1440 \times 0.104 = 149$ liters per 24 hours. The 65 mM of PO_4 excretion (= 2 gm. of P) is the maximum likely to be found in the human 24 hour urine.

Free H_2CO_3, unlike $BHCO_3$, appears not to be "actively" reabsorbed, but merely to pass back by diffusion into the blood with reabsorbed water. There is no physiological need for H_2CO_3 reabsorption, since CO_2 is continually produced by combustion in the body. Evidence that H_2CO_3 reabsorption is never "active" in the sense that the concentration in the tubular lumina is lowered below that in the blood, is seen in the fact that the CO_2 tension in the urine never, according to our results, is found below that in the arterial blood.

The changes in $BHCO_3$ and H_2CO_3 which acidify the urine must also affect the acid-salt ratios of other buffers. Of these the most important are normally the phosphates (Peters and Van Slyke, 1931, pp. 959 and 1111). With alkaline phosphate the reversible reaction

$$(4) \qquad H_2CO_3 + B_2HPO_4 \rightleftarrows BHCO_3 + BH_2PO_4{}^{*}$$

must occur until the relationship indicated by Equation 5 is established.

$$(5) \qquad \frac{[B_2HPO_4]}{[BH_2PO_4]} = 0.2\,\frac{[BHCO_3]}{[H_2CO_3]}$$

(The factor 0.2 is the approximate ratio of the second dissociation constant of H_3PO_4 to the first dissociation constant of H_2CO_3.)

It appears that all the phosphate excreted can be changed in the tubules from B_2HPO_4 to BH_2PO_4 by the reaction of Equation 4 if the following conditions are met: (1) The $BHCO_3$ formed by the reaction must be absorbed, so that the reaction can continue from left to right. (2) At least 1 mole of H_2CO_3 per mole of B_2HPO_4 must be present in the initial glomerular filtrate. (3) The H_2CO_3 in the filtrate must not be diminished (by absorption) below this proportion until the reaction and the reabsorption of $BHCO_3$ are completed. It appears possible that all these conditions may be met. (1) As indicated by Table V, reabsorption of $BHCO_3$ in urine of ordinary acidity is nearly complete. (2) There is more than 1 mole of H_2CO_3 per mole of B_2HPO_4 present in the calculated filtrate. (3) Concerning the relative reabsorption velocities of H_2CO_3 and $BHCO_3$ there are no data. Since about 20 times as many moles of $BHCO_3$ as of H_2CO_3 are reabsorbed, however, it appears quite possible that reabsorption of $BHCO_3$ may sufficiently outrun that of H_2CO_3 to meet the third condition.

The acidifying effect of $BHCO_3$ reabsorption may be reenforced by B_2HPO_4 reabsorption, since only part of the glomerular PO_4 reaches the urine (Table V). However, to shift the B_2HPO_4:BH_2PO_4 ratio of 4:1 in the glomerular filtrate to the value of practically 0 in urine of pH 5, by B_2HPO_4 absorption alone, would require reabsorption of 80 per cent of the filtrate PO_4 (all the B_2HPO_4). It does not appear from Table V that phosphate reabsorption always proceeds to this extent. Hence, at times, in

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[*] This reaction, with tubular absorption of the $BHCO_3$, was originally proposed by Cushny (1926) as a factor in acidification of urine.

any case, part of the decrease in the $B_2HPO_4:BH_2PO_4$ ratio, which occurs as the filtrate passes down the tubules, is apparently due to other acidifying causes, such as $BHCO_3$ reabsorption and the consequent reactions. Under no condition could B_2HPO_4 reabsorption, by reversal of the reaction (Equation 4), have much effect in decomposing filtrate $BHCO_3$, because the amount of the latter is so immensely greater.

If the change from the alkaline to the acid form of phosphate during passage of the tubules is accomplished by reaction of the alkaline form with H_2CO_3 (Equation 4) and reabsorption of the $BHCO_3$ formed, a similar process must occur with the other urinary buffers. The condition under which the greatest demand is put upon such a mechanism is presumably that of severe diabetic acidosis, in which large amounts of free hydroxybutyric acid are added to the acid phosphate in the urine. However, the maximum amount of total titratable acid (free organic acid plus acid phosphate) found by Stillman, Van Slyke, Cullen, and Fitz (1917) in severe diabetic acidosis was 120 mM per 24 hours. This is not beyond the possibility of production by reaction of sodium hydroxybutyrate with the 195 mM of H_2CO_3 calculated in Table V to be present in the glomerular filtrate, the resultant $BHCO_3$ being reabsorbed, and the free organic acid left in the urine. Acidification by reabsorption of alkali salts of hydroxybutyric acid can hardly be important, because, to lower the pH to 5 by this means would require reabsorption of about 98 per cent of the hydroxybutyrate in the glomerular filtrate, and the large amounts excreted (up to 75 grams in the 24 hour urine) preclude such complete reabsorption.

Besides reabsorption of $BHCO_3$ and B_2HPO_4, an alternative method of acidifying the urine, and preserving the alkali reserve of the body, would be the excretion of free HCl into the tubular lumina (no other anion than Cl^- in the normal blood could provide sufficient acid to react with the amount of $BHCO_3$ estimated to be in the glomerular filtrate). Similarly one could assume in diabetic acidosis the tubular excretion of free hydroxybutyric acid. However, the fact that the pH of the urine, unlike that of the gastric juice, is never below 4.8, an acidity which could be produced by either H_2CO_3 or BH_2PO_4 left after reabsorption of $BHCO_3$ and B_2HPO_4, inclines one to favor the hypothesis that reabsorption of the alkaline buffer salts produces the acidification of the urine. (In a solution under 67 mm. of CO_2 tension, with $BHCO_3$, and hence B_2HPO_4 completely removed, the pH would be about 4.4.) Control of the relative speed and completeness with which $BHCO_3$ and H_2CO_3 are reabsorbed appears to be sufficient to control the entire observed range of urinary acidity and alkalinity.

The mechanism by which ammonia, formed in the kidney (Nash and Benedict, 1921), causes *excretion of acids as ammonium salts,* without loss of fixed base from the body, is also explicable by active reabsorption of $BHCO_3$. The excretion can be explained by assuming that NH_4HCO_3 formed in the kidney is filtered together with BA brought by the blood, and that in the tubules ions are interchanged, $BHCO_3$ is reabsorbed, and NH_4A is left in the urine. (A = anion of hydrochloric, hydroxybutyric, or other acid excreted as ammonium salt.)

Formation of Alkaline Urine—When plasma bicarbonate concentration is raised above a level of about 31 mM, the urine usually becomes alkaline (Palmer and Van Slyke, 1917). There is in such urine a relatively high concentration of $BHCO_3$ (see Gamble (1922); Marshall (1922); also data in this paper). Excretion of $NaHCO_3$ may reach several grams per hour. The glomerular filtrate of about 6 liters per hour, if also of more than 31 mM $NaHCO_3$ concentration, would contain over 20 grams of $NaHCO_3$ per hour. Hence the observed maximal urinary bicarbonate excretion, of at most a fraction of this amount, can be accounted for merely by incomplete reabsorption of the filtered $BHCO_3$. The relatively large concentrations of $BHCO_3$ in the urine are sufficient to cause the maximal observed pH values, about 8, and by interaction with the phosphates and other buffers, would increase the urinary ratios of their alkali salts to their free acids or acid salts.

SUMMARY

A technique is described for measurement of CO_2 tension in urine. The urine is equilibrated at 38° with 0.02 its volume of an air-CO_2 mixture, and the CO_2 tension is determined by microanalysis of the gas bubble.

The method has been checked by determinations on urines in which the CO_2 tensions were set at levels known within 0.2 mm.

The error of the method averaged 1 mm., and in no case exceeded 3 mm.

CO_2 tensions of human urines drawn from the bladder, in both normal and pathological cases, were found over the range from 40 to 200 mm. The majority were between 45 and 95 mm. The greatest accumulation was within the zone 67 ± 5 mm., within which one-fourth of the tensions was found.

CO_2 tensions above 80 mm. were found only in urines sufficiently concentrated to have specific gravities of 1.020 or higher. Tensions above 120 mm. were frequently found after severe exercise, and after taking sodium bicarbonate. Such high tensions, however, were not observed in any case in which the urine had been retained in the bladder longer than 4 hours.

The fact that urine tensions below 40 mm. were never encountered makes it appear doubtful that the CO_2 tension of the urine ever falls below that of the arterial blood.

Our results confirm Gamble's (1922) in showing that CO_2 tension and H_2CO_3 concentration in urine remain within the same range, regardless of variations in total CO_2 and $BHCO_3$ content, so that high $BHCO_3$ content tends to produce urine of high pH (Fig. 5). Our data differ from Gamble's only in showing, because of wider CO_2 tension range, somewhat less degree of correlation between $BHCO_3$ and pH.

The results are compatible with the filtration-reabsorption theory of urine excretion. The entire observed ranges of urinary CO_2 tension, CO_2 content, and pH can be quantitatively deduced from reasonably probable variations in reabsorption of $BHCO_3$ and H_2CO_3 from the glomerular filtrate.

BIBLIOGRAPHY

Austin, J. H., Cullen, G. E., Hastings, A. B., McLean, F. C., Peters, J. P., and Van Slyke, D. D., *J. Biol. Chem.*, **54**, 121 (1922).
Cushny, A. E., The secretion of urine, London, 206 (1926).
Fredericq, L., *Arch. internat. physiol.*, **10**, 391 (1910).
Gamble, J. L., *J. Biol. Chem.*, **51**, 295 (1922).
Hastings, A. B., Sendroy, J., Jr., and Robson, W., *J. Biol. Chem.*, **65**, 381 (1925).
Jolliffe, N., Shannon, J. A., and Smith, H. W., *Am. J. Physiol.*, **104**, 639 (1932).
Mainzer, F., and Bruhn, M., *Biochem. Z.*, **230**, 395 (1931).

Mainzer, F., and Shen, C. T., *Arch. ges. Physiol.*, **222**, 1 (1929).

Marshall, E. K., Jr., *J. Biol. Chem.*, **51**, 3 (1922).

Møller, E., McIntosh, J. F., and Van Slyke, D. D., *J. Clin. Inv.*, **6**, 427 (1928).

Nash, T. P., Jr., and Benedict, S. R., *J. Biol. Chem.*, **48**, 463 (1921).

Palmer, W. W., and Van Slyke, D. D., *J. Biol. Chem.*, **32**, 499 (1917).

Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Baltimore, **1** (1931); **2** (1932).

Pflüger, E., *Arch. ges. Physiol.*, **6**, 69 (1872).

Richards, A. N., Methods and results of direct investigations of the function of the kidney, Baltimore (1929).

Sendroy, J., Jr., Seelig, S., and Van Slyke, D. D., *Sunti comunicaz. scient., XIV cong. internaz. fisiol.*, Rome 232 (1932); *J. Biol. Chem.*, **106**, 463 (1934).

Shannon, J. A., Jolliffe, N., and Smith, H. W., *Am. J. Physiol.*, **104**, 534 (1932).

Stillman, E., Van Slyke, D. D., Cullen, G. E., and Fitz, R., *J. Biol. Chem.*, **30**, 405 (1917).

Strassburg, S., *Arch. ges. Physiol.*, **6**, 65 (1872).

Van Slyke, D. D., Sendroy, J., Jr., and Liu, S. H., *J. Biol. Chem.*, **95**, 547 (1932).

Wearn, J. T., and Richards, A. N., *Am. J. Physiol.*, **71**, 209 (1924).

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OBSERVATIONS ON THE ETIOLOGY AND TREATMENT OF ANEMIA ASSOCIATED WITH HOOKWORM INFECTION IN PUERTO RICO[1]

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[1] These observations were carried out in 1931 by the Commission of the Rockefeller Foundation for the Study of Anemia in Puerto Rico.

I. INTRODUCTION

Since the demonstration of the widespread occurrence of hookworm infection in the Southern United States by Stiles (1), Harris (2), and others (3) (4), the presence of hookworms in the intestinal canal of thousands of anemic children and adults in certain areas of the New World has been established. Although the exact causal relationship of the parasite to the anemia has not been made clear, hookworm disease in regions of severe infection has been regarded in a general way as synonymous with anemia when the latter was not due to other obvious causes. The very name given to the parasite, *Necator americanus* (American murderer), symbolized the general belief that in this widespread anemia a living parasitic agent was primarily the cause of disability and death. Although certain observations have been made which do not conform entirely to the simplicity of this idea, only in the past decade has the relationship of defects of nutrition to certain types of anemia been demonstrated in any quantitative manner. Recently the idea of a relationship between malnutrition and the degree of anemia in hookworm disease has been suggested by Smillie (5), Schapiro (6) and others.

The influence of these two points of view concerning the etiology of the anemia of hookworm disease may be seen to advantage in the history of anemia in Puerto Rico, where it has long been noted to be a common affliction. In 1788 Abbadd (7) stated "The jibaros (peasants) lacked liveliness and had the color and aspect of convalescents." In 1883 Del Valle (7) remarked "It is only the exception to see one of good ruddy color." At this time, according to Ashford (8), the anemia was commonly believed to be due to faulty diet. Therefore, the popular conception, although not technically so expressed, was that of a dietary deficiency anemia. Somewhat later as generous food with relatively small doses of iron and arsenic apparently failed to cure, the condition was attributed to malaria, climate or lack of hygiene. In 1888 Blanchard (8), referring to the finding of hookworms in other West Indian islands and in Brazil, suggested that the parasite was probably present in Puerto Rico as well. In 1899 Ashford (7) discovered the parasite in Puerto Rico. Five years later Ashford, King, and Igaravídez (8) published an extensive report of their observations upon "anemia" in Puerto Rico. In the report they stated, "We have seen that the major portion of this 'anemia' is uncinariasis, and we earnestly recommend to our professional brethren . . . . that no further reliance be placed on iron, quinine, and tonics in general. . . . ." They also reported the failure of improved diets in the relief of anemia. In this way "anemia" in Puerto Rico, as in other regions of hookworm infection, became "uncinariasis." The study of the problem then became for several years the study of appropriate measures against the parasite.

However, in 1929, perhaps influenced by the recent changes in point of view toward the mechanism of anemia elsewhere, Suárez, Otero, and Molina (9) stated in a report of observations upon two cases of anemia that the most important factor in the production of anemias in Puerto Rico was the inadequate diet. It was only then emphasized that Blaud's pills had long been used in Puerto Rico as an adjunct to measures against the hookworm. Moreover, in 1931 Suárez (10) observed a reticulocyte response in a case of hookworm anemia following the administration of an aqueous extract of liver. Although unaware of these particular facts, it seemed worth while to us to attempt to resurvey the problem of the anemia of hookworm disease in Puerto Rico in the light of recent observations upon the etiology and treatment of similar types of hypochromic anemia not associated with the hookworm. To the attack upon the disease by the elimination of the parasites and by the prevention of their spread, it was hoped that a third method might be added—the prevention or treatment of the anemia per se. It was our good fortune to be able to carry out the observations described below, of which a preliminary report has already been published (11), under the auspices of the Rockefeller Foundation in 1931.

II. METHODS

Studies were made of 83 patients with anemia and hookworm infection. The patients, who were mostly from rural districts, were observed either in various hospitals or as regular visitors to a special clinic established at the Presbyterian Hospital in San Juan. The patients were primarily selected because of the severity of their anemia and the absence of complicating infections or sources of blood loss. Children, as well as adults of both sexes, were included in the series. In addition, field observations were carried out by Miss Celia Nuñez at Cidra, a small agricultural community, upon 32 patients, both children and adults, whose anemia was associated with hookworm infestation. The methods and results of this work, by permission of Miss Nuñez, are briefly described below, and are to be published elsewhere in full (12).

Patients under observation in the hospitals, unless otherwise specified, were maintained on a basal diet resembling as closely as possible the usual food of the Puerto Rican peasants. The major articles of this diet were rice, beans, and white bread, such tropical vegetables as yautiá, ñame and plátano, and fruit juices. Meat and eggs were not given. A small amount of milk was allowed in coffee. Salt and sugar were given as desired. Carbohydrate desserts with syrup were freely used. The quantity of the diet was limited only by the desire of the individual for food. In the management of out-patients, suggestions as to diet were never made. In as many instances as possible a general history and diet history were taken, and a physical examination was performed. The blood was examined for malaria in all doubtful cases, and the Kahn test was performed in all instances. The presence of hookworm was assured by direct examination of the stool for ova. In certain instances counts of the ova in the stools were made by the Stoll technique. In others all the worms expelled by efficient anthelmintics were collected. These essential and time-consuming observations were kindly performed by Dr. Florence King Payne. On 54 patients gastric analyses were performed.

The hematological studies, except in the field experiment referred to above, were made from 5 cc. samples of venous blood drawn without stasis and rendered incoagulable by 0.05 cc. of a 20 per cent solution of potassium oxalate. Since the dilution and cell shrinkage factor was small and constant, no correction was made for it. The corpuscular counts were made by the usual technique with certified apparatus. The hemoglobin percentage was determined with a single Sahli instrument with a solid standard. One hundred per cent of hemoglobin was considered equivalent to an oxygen capacity of 21 volumes per cent. The mean corpuscular volume and hemoglobin indices were calculated from hematocrit values determined by the method of Wintrobe (13). The serum color was estimated against bichromate standards by the method of Murphy (14) and the serum bilirubin was determined by the quantitative Van den Bergh method. Blood films were made upon cover glasses and supravitally stained for reticulocytes with brilliant cresyl blue before being counterstained with Wright's stain. When patients were in hospital, a 5 cc. sample of venous blood was usually taken every other day, and reticulocyte counts were made daily from capillary or venous blood as was most convenient. Out-patients usually had venous blood samples examined every one or two weeks. Biopsies of the sternal bone marrow were made upon 15 patients at various stages of their anemia.

Aside from these purely descriptive observations, the general plan of study was to observe under controlled conditions the effect, especially upon blood formation, *of a single procedure.* Thus, individually, the effect was observed of removal of the parasites of the administration of a high-protein diet, of various extracts of liver and of iron salts. The immediate effect of these procedures upon blood formation was determined by daily observations of the reticulocyte percentage, a method which has been applied to the study of both macrocytic and hypochromic anemias (15) (16). In addition, the effect of any given procedure upon the red blood cell and hemoglobin values was determined. Certain of the therapeutic procedures mentioned above were carried out in the field experiment at Cidra, where observations of the red blood cell and hemoglobin values were made upon capillary blood.

III. OBSERVATIONS CONCERNING THE ETIOLOGY OF THE ANEMIA

A. Morphological considerations. At the same time that the study of the anemia of hookworm disease was under way, observations were being made upon sprue as well as upon other types of anemia encountered in the same community. It became apparent that in certain instances patients with hookworm ova in the stools presented the clinical and hematological picture of sprue. Conversely, patients diagnosed clinically as sprue, sometimes presented a blood picture indistinguishable from that associated with severe hookworm infestation. These facts indicated that it was not easy to discriminate between the two conditions in all instances. Furthermore, several patients with no hookworm infection were observed to show blood pictures and hematological responses identical with those of patients with hookworms. The 83 patients forming the basis of this report were thus of necessity selected somewhat arbitrarily because they showed a severe degree of hypochromic anemia and had hookworm ova in the stools.

1. The blood picture. The blood findings in the 83 patients on first coming under observation are summarized in table 1. The outstanding features of the anemia were the reduction of the average cell size and hemoglobin concentration. Thus, the hemoglobin values were reduced in proportion to the number of red blood cells. Twenty-four patients had a color index of less than 0.5; only 9 had a color index of over 0.7. The severity of the anemia of the patients selected may be judged from the fact that only 5 patients had initial hemoglobin values of 50 per cent or over. Of the remaining 78 patients the initial hemoglobin values were less than 10 per cent in 3, between 10 and 19 per cent in 7, between 20 and 29 per cent in 26, between 30 and 39 per cent in 30, and between 40 and 49 per cent in 12 patients. According to Wintrobe, the average normal value for the mean corpuscular erythrocyte volume is 87 cubic micra with limits of 80 and 94 cubic micra. In this series, 7 patients had initial values within normal limits, but the majority showed a distinct reduction. Fifteen patients had mean corpuscular volumes of 60 cubic micra or less when first observed. Under controlled conditions the reticulocytes were usually less than 3 per cent, but in some patients without therapy reticulocyte counts varying from 3 to 6 per cent were found over periods of several days. Rarely an isolated higher value was noted. Nucleated red blood cells of the normoblast type occasionally were seen in the blood films of the patients with the more severe degrees of anemia. The number of leucocytes was usually within normal limits or slightly increased. Occasionally leucopenia with relative lymphocytosis was observed. Eosinophilia was common. Platelet counts were not made, but the impression gained from observation of blood films was that no significant variation from the normal existed. The icteric index was with few exceptions below 6, the normal value for the method. This fact was confirmed by the quantitative Van den Bergh test.

TABLE 1

The blood findings, number of ova or hookworms, results of gastric analyses, and incidence of glossitis and diarrhoea

CASE NUMBER	AGE	SEX	RED BLOOD CORPUSCLES	HEMOGLOBIN	WHITE BLOOD CORPUSCLES	RETICULOCYTES	ICTERIC INDEX	COLOR INDEX	MEAN CORPUSCULAR VOLUME	OVA PER CENTIGRAM	NUMBER OF WORMS	GASTRIC ANALYSES*	GLOSSITIS	DIARRHOEA
1	9	M.	3.91	35	7.8	1.6	2	0.45	55.4	+				
2	10	F.	1.83	21	6.8	4.9	2	0.57	66.6	281		Alc.	0	+
3	35	F.	2.22	37	7.3	5.6	1	0.83	85.1	+				
4	33	F.	3.16	35	3.4	1.0	2	0.55	81.4	+				
5	35	M.	3.63	50	5.5	1.4	2	0.69	87.2	50		Alc.	0	++
6	21	F.	4.58	54	4.2			0.59	65.0	112		Alc.	0	0
7	15	F.	4.14	44	8.0	0.6	3	0.53	60.6	+		0		
8	22	M.	2.53	32	6.9	5.2	2	0.63	73.7	357		Alc.	0	±
9	25	M.	2.36	29	4.6	2.7	10	0.61	69.0	201		Hist.	0	0
10	45	M.	1.93	27	8.3	2.4	4	0.70	84.8	+			0	0
11	18	M.	2.29	37	4.7	1.9	2	0.81	56.0	119		Alc.	0	+
12	12	F.	2.85	56	5.3	0.4	6	0.98	97.3	+	102	Hist.		
13	48	M.	3.53	31	5.4	2.0	4	0.44	55.7	+	1,098	0	0	+
14	25	M.	3.76	46	6.6	0.8	3	0.61	64.8	2		0		
15	32	M.	2.48	28	14.1	2.6	1	0.56	65.3	+				
16	12	F.	2.42	22	11.0	1.8	4	0.45	57.6	44		Alc.		
17	12	M.	0.95	10	11.4	2.4	4	0.53	65.2	+	630	Alc.		
18	10	M.	2.37	22	6.8	2.6	3	0.46	63.2	+	344	Alc.		
19	20	F.	2.67	22	9.6	1.4	1	0.41	52.4	394				
20	6	F.	2.80	23	5.7	2.2	3	0.41	63.2	4		Alc.	0	+
21	22	M.	3.60	28	6.5	1.6	1	0.40	54.1	+				
22	19	F.	2.11	18	7.6	2.4	2	0.43	57.8	+		Alc.	0	0
23	51	M.	2.14	24	3.8	1.2	3	0.56	70.0	48		Alc.	0	0
24	20	F.	3.78	49	6.4		2	0.65	67.5	+				
25	40	F.	3.07	50	8.9	1.6	3	0.81	83.3	+		Hist.	+	++
26	9	M.	1.22	9	6.9	0.6	2	0.37	61.4	494			0	+
27	30	M.	2.09	22	1.9	6.4	4	0.53	70.9	286		Hist.		
28	42	F.	3.98	59	6.1	1.0	3	0.74	76.1	+				
29	55	M.	2.91	25	5.6	2.6	2	0.43	57.0	158		Hist.	0	+
30	10	M.	0.86	9	7.0	2.4	2	0.52	63.7	1,307		Alc.	0	0
31	42	M.	3.12	36	5.9	1.0	2	0.58	65.4	625				
32	13	M.	2.00	19	3.0	1.8	2	0.48	55.5	918		Alc.	0	0
33	60	M.	3.03	35	5.6	1.6	2	0.58	64.3	109			+	0
34	63	M.	3.40	43	11.2	1.8	1	0.63	67.6	2		Alc.	0	+
35	16	M.	3.18	46	5.1	2.8	3	0.72	74.2	+		Alc.	0	0
36	10	M.	1.76	18	7.2	0.8	2	0.51	60.2	130		Hist.		
37	11	M.	0.78	8	7.1	2.2	1	0.51	83.3	498		Hist.	0	+
38	9	M.	3.29	42	12.7	5.4	3	0.64	68.1	+				
39	40	M.	1.91	17	2.9	2.4	2	0.45	62.1	375		0	0	0
40	37	F.	2.71	23	4.3	1.6	2	0.42	54.2	362				
41	63	M.	3.53	35	8.0	2.2	3	0.50	61.5	161		0	+	+
42	42	F.	2.58	25	5.4	0.8	2	0.48	54.8	+				

TABLE 1—*Concluded*

CASE NUMBER	AGE	SEX	RED BLOOD CORPUSCLES	HEMOGLOBIN	WHITE BLOOD CORPUSCLES	RETICULOCYTES	ICTERIC INDEX	COLOR INDEX	MEAN CORPUSCULAR VOLUME	OVA PER CENTIGRAM	NUMBER OF WORMS	GASTRIC ANALYSES*	GLOSSITIS	DIARRHOEA
43	45	M.	3.59	35	5.0	1.2	4	0.49	59.7	132		0	0	++
44	50	F.	2.33	23	5.4	1.0	2	0.49	64.8	484			0	++
45	19	M.	2.90	28	15.8	1.6	3	0.48	63.0	360			0	0
46	6	F.	4.25	41	9.2	0.8	2	0.48	53.8	+				
47	10	M.	1.93	26	12.7	9.0	3	0.67	73.5	307				
48	60	M.	2.49	30	7.2	3.4	2	0.60	74.5	306		Alc.	0	+
49	40	M.	2.64	24	6.9	3.0	2	0.45	64.8	+	313	Alc.	+	++
50	12	M.	3.80	38	11.8	2.8	4	0.50	63.4	182			0	0
51	14	M.	3.07	34	7.9	2.2	3	0.55	61.2	250		Alc.		
52	30	M.	2.70	25	6.8	1.0	3	0.46	63.7	655	76+	0	+	+
53	35	M.	2.03	28	5.5	0.8	3	0.69	76.8	931	2,231	Alc.	0	0
54	35	M.	3.66	38	7.7	0.8	3	0.52	64.2	+			0	0
55	32	M.	2.97	31	7.7	2.0	4	0.52	64.2	402				
56	17	F.	3.53	36	7.3	1.4	3	0.51	63.7	+		0	0	0
57	20	M.	3.48	43	9.6	1.2	1	0.62	66.5	+	562	Alc.		
58	38	M.	2.51	25	6.3	4.6	2	0.50	55.6	113	155	Alc.	0	0
59	35	M.	2.43	25	6.4	2.4	4	0.51	71.1	+	1,066	Alc.		
60	12	M.	1.82	20	9.2	3.8	2	0.55	66.9	+	718	Alc.		
61	14	M.	2.02	21	2.8	2.4	4	0.52	61.8	101		0		
62	52	M.	2.14	32	4.4	3.0	3	0.77	68.4	607	1,424	Alc.	0	0
63	19	M.	4.38	38	8.9	1.8	2	0.43	58.2	112	103	Hist.		
64	68	M.	3.57	44	7.4	5.2	2	0.61	71.5	118			0	0
65	18	M.	1.57	22	6.8	1.4	2	0.69	78.4	601		0	+	0
66	40	M.	2.55	35	5.3	0.7	2	0.69	75.6	650	723	Hist.	0	+
67	35	M.	2.60	36	3.1	1.0	2	0.69	76.2	+	724	0		
68	18	M.	1.98	27	10.0	2.8	4	0.68	70.2	+	1,234	Alc.	0	+
69	17	M.	2.88	33	3.8	1.0	3	0.67	67.9	30		Hist.	0	0
70	20	F.	2.77	39	7.1	3.6	2	0.72	73.2	+	1,106	Alc.		
71	19	F.	2.07	37	6.4	1.1	1	0.89	91.8	+		Alc.	+	+
72	18	M.	2.62	19	3.0	1.8	4	0.59	72.7	179		0	0	0
73	28	M.	3.00	36	6.6	0.8	5	0.60	65.6	+				
74	35	M.	3.54	34	4.8	1.6	3	0.48	59.0	+		Alc.	0	0
75	34	M.	2.80	39	4.3	2.2	3	0.70	77.1	+		Alc.	+	+
76	25	M.	4.12	45	6.5	1.0	3	0.55	65.0	+				
77	6	F.	1.77	17	12.1	0.8	3	0.48	59.8	302			0	+
78	15	M.	3.63	34	9.2	1.4	2	0.48	62.7	49				
79	35	F.	3.49	36	4.7	2.0	8	0.52	64.2	+			0	0
80	18	F.	3.68	41	9.2	0.4	3	0.56	62.0	152				
81	24	F.	3.53	38	6.8	3.2	2	0.54	51.4	+	40	0		
82	30	F.	2.88	37	4.5	3.6	4	0.64	66.1	124		Hist.	0	+
83	18	F.	3.71	46	5.7	1.8	4	0.62	63.5	+		Alc.	0	0

* *Alc.* indicates secretion of free HCl after ingestion of 50 cc. of 7 per cent alcohol; *hist.* after injection of 0.5 mgm. of histamine phosphate; 0 indicates no secretion of free HCl after histamine injection.

With the exception of the mean corpuscular volume determinations in these patients, nothing new is added to the description of the blood picture of the hypochromic anemia of hookworm disease. The blood picture in this series of patients was that of a hypochromic anemia, usually microcytic, without evidence of active blood regeneration. It thus differs in no obvious way from the blood picture of types of hypochromic anemia not associated with hookworm infestation encountered in Puerto Rico and elsewhere.

2. The bone marrow. The bone marrow findings are also in agreement with those found in other types of hypochromic anemia. Piney (17) has shown that only the ribs, flat bones, and vertebral column normally contain active cellular marrow in adult life. Peabody (18) has emphasized the fact that the pathological process present in the normally cellular areas does not necessarily extend to the long bones. For these reasons biopsies for histological examination of the bone marrow were performed on the sternum. A specimen of marrow was removed from this site at biopsy at various stages of the anemia in 15 patients, and the tissue subjected to histological study.

The sections were moderately but quite definitely more cellular than the normal marrow. The degree of increased cellularity varied in each section and varied from patient to patient. The number of fat cells was decreased and vascularity was marked. Islands of young cells were present with pale, clear cytoplasm and round to oval nuclei containing a few small, discrete masses of chromatin. These cells were considered to be at a stage not far removed from hematopoietic vascular endothelium. A second cell type was seen, although not in large numbers. This was a small round cell with a narrow rim of cytoplasm and a nucleus containing large masses of heavily stained chromatin. These cells were considered to belong to a primitive pluripotential cell type. The predominant cell type, however, was the normoblast. Large numbers of these cells were present, both in islands and diffusely scattered throughout the tissue. There was a sharp disproportion between erythroblastic cells and those of the granulocyte series. Whereas this ratio is about equal in normal marrow, here the former outnumbered the latter from three to five times. The small number of granulocytes present showed no disturbance of maturation.

The histological picture described differs from the normal and from that of the marrow in cases of severe anemia due to toxic or infectious conditions such as nephritis of severe grade. It resembles to a certain extent the findings of other investigators of hookworm disease. Ashford and Igaravídez (7) reported the finding of many normoblasts and eosinophils. Day and Ferguson (19) occasionally found in addition a gelatinous appearance in the femoral marrow of autopsied patients. Dameshek (20) and others have reported characteristic pathological changes in the bone marrow removed at biopsy from the sternum of cases of idiopathic hypochromic anemia. The marrows are stated to be more cellular than normal, with less fat, and to show an increase in the ratio of erythroblastic to leucocytic cells. Since the histological picture in the bone marrow of the hypochromic anemia of hookworm infestation thus apparently closely resembles the bone marrow picture in hypochromic anemia not associated with hookworm infestation, the common genesis of both types of anemia is suggested. These observations will be discussed in detail in a later communication.

B. Physiological considerations. The belief that the hookworm is the dominant factor in producing the anemia associated with it deserves careful study because, although it has been so often expressed and generally accepted, no entirely satisfactory explanation of its method of action has yet been given. For this reason consideration was given to the ways in which the parasite, alone or as one of several factors, could conceivably have a relation to the anemia.

1. The relation of the hookworm to the anemia. Although in general there is stated to be a relationship between the degree of anemia and the number of worms harbored, attempts at correlation of the number of parasites with the degree of anemia in individual cases have met with no particular success in the hands of others (5) (6) (21) (22) (23). This feature of the analysis was accordingly not exhaustively pursued. However, in 17 patients all the stools were saved and worm counts were made during elimination of the parasites with hexylresorcinol by the technique of Lamson (24). In figure 1 is shown the relationship for 17 individuals between the number of parasites discharged and the initial hemoglobin level. It is clear that there is no obvious correlation. On the same chart are also plotted for 55 individuals the average number of ova in a centigram of feces, corrected for the consistency of the material, against the initial hemoglobin value. Again there is apparently no significant correlation to be observed. In this respect, then, these observations are in agreement with other reports and clearly suggest the influence of factors other than the hookworm. The possibility of a contributing influence nevertheless remains.

a. The possible toxic effects of the hookworms. Although Whipple (25) has demonstrated weak hemolytic factors in the hookworms of men and dogs, convincing evidence for the rôle of hemolysis in the production of this anemia is lacking. The morphological characteristics of the anemia are not those of a hemolytic process, such as is illustrated by hemolytic jaundice, in which increased numbers of reticulocytes and increased serum color are constantly present. It is, of course, possible that such a condition, if originally present, might later be hidden beneath the impress upon the blood picture given by the failure of the bone marrow. However, with the exception of

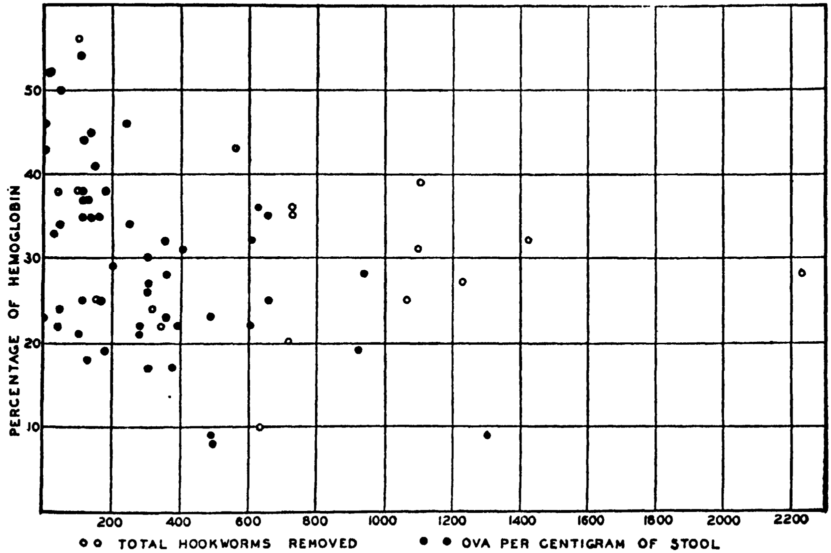

FIG. 1. RELATIONSHIP BETWEEN THE ORIGINAL PERCENTAGE OF HEMOGLOBIN AND THE NUMBER OF WORMS SUBSEQUENTLY REMOVED FROM 17 PATIENTS, AND BETWEEN THE ORIGINAL PERCENTAGE OF HEMOGLOBIN AND THE AVERAGE NUMBER OF OVA PER CENTIGRAM OF STOOL OF 55 PATIENTS

malaria or of a septicemia, particularly that due to the streptococcus, the action of infection is not that of a hemolytic process but of an inhibition of bone marrow function. De Langen (26) champions this theory; and Fülleborn (27) states that the hookworm secretes a substance which has a toxic effect on the bone marrow. If, then, the presence of hookworms has a dominant effect in depressing the bone marrow activity, two phenomena should be demonstrable. First, the removal of the parasites should produce increased blood regeneration, and secondly, the presence of the parasites should inhibit, to a certain extent at least, the influence of agents capable of promoting blood formation. Observations designed to test the validity of these points were accordingly made.

The effect of the removal of the hookworms upon blood production. Twelve patients were kept in hospital upon the basal diet referred to above, and no form of therapy for anemia was given. After a control period of a few days the parasites were removed by means of hexylresorcinol (24) and the effect upon reticulocyte production and red blood cell and hemoglobin values observed. In table 2 are shown the effects upon the hemoglobin and red blood cell values and the number of hookworms removed in each instance. It is evident that during periods of time as long as twenty-eight days after the first treatment for hookworms, very little effect upon red blood cell or hemoglobin values was produced. The average gain in red blood cells in 9 patients (calculated by extrapolation in cases 59, 67, and 81) was only 0.48 million per cubic millimeter in twenty-eight days. In two instances, cases 52 and 63, the red blood cells actually declined slightly in number. The effect upon hemoglobin production was even less striking the average increase in 9 patients being only 4 per cent in twenty-eight days. In two instances, cases 59 and 61, the hemoglobin percentage actually became less. During six weeks the average gain in red blood cells of 7 patients was 0.49 million per cubic millimeter and in hemoglobin was 3 per cent. It is thus clear that the removal of the parasites did not bring about any striking increases in red blood cells and hemoglobin, and thus affords no evidence for the dominant inhibitory effect of the hookworms on bone marrow function. It is important to realize, moreover, that the fact that slight increases in hemoglobin and red blood cell values occurred is not necessarily evidence of increased activity on the part of the bone marrow. Precisely such a result would occur if the amount of blood loss were diminished.

In the light of the observations of Minot and his collaborators (15) (16), as well as from the work of others, the value of the study of reticulocyte production as a measure of bone marrow activity has been clearly established for both macrocytic and hypochromic anemias. This phenomenon should, theoretically at least, be applicable to the problem of detecting whether the removal of possible "toxic inhibition" of the bone marrow caused by the presence of the hookworms would result in increased hematopoietic activity. The principle is best illustrated by the effect of removing the parasites in another tropical disease associated with anemia. The anemia of many cases of malaria is clearly due, at least in part, to the rapid blood destruction dependent upon the activities of the plasmodia. The blood picture is the reverse of that of hookworm disease, since it is characterized by signs of rapid destruction and production of red blood cells, best shown by increases of the serum bilirubin and by persistently elevated reticulocyte counts during the active stages of an early infection. However, it is possible to demonstrate that in addition to the hemolytic process there is, as with other types of infection associated with fever, an actual inhibition of bone marrow function. This is shown by the fact that shortly after the abolition of the fever with quinine an increase of the reticulocytes in the peripheral circulation takes place. This temporary increase can easily be demonstrated in suitable cases of malaria, and is best explained on the basis of the removal of an inhibitory effect.

In figure 2 is shown the contrast between the effect of removing the parasites in a patient with malaria and in a patient with hookworm infestation, case 52. Both patients were severely anemic. In the former, the distinct temporary increase of reticulocytes during ten days following treatment with quinine is in sharp contrast to the absence of any such effect resulting from the elimination of the hookworms in the latter patient. If the hemolytic function alone had been abolished by the removal of the malaria parasite, the reticulocytes would have declined steadily without the temporary increase. In table 2 are shown the results of hookworm removal upon reticulocyte production in 12 patients, including case 52. It is clear that in no instance was there a significant increase of reticulocytes. In con-

TABLE 2

Comparison of effects upon hematopoiesis of removal of hookworms and of administration of ferric ammonium citrate to patients upon basal die

First periods. Hookworms removed with hexylresorcinol*

	CASE 13			CASE 18			CASE 49			CASE 52		
	Red blood cells	Hemo-globin	Reticulo-cytes	Red blood cells	Hemo-globin	Reticulo-cytes	Red blood cells	Hemo-globin	Reticulo-cytes	Red blood cells	Hemo-globin	Reticulo-cytes
days	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*
0	3.39	33	1.6	2.37	20	4.0	2.70	26	1.6	2.40	27	0 6
2	3.26	34	2.6	2.05	21	4.8	2.81	26	1.4	3.01	30	1.0
4	3.21	32	1.8	1.74	20	6.8	3.10	28	2.8	2.68	25	1.0
6	3.20	35	1.0	2.37	20	5.7	3.30	30	3.8	2.98	30	0.8
8	3.31	43	2.8	1.96	21	4.8	3.20	32	2.8	3.00	26	1.4
10	3.46	36	2.0	2.03	20	2.4	3.39	29	1.4	2.67	55	1.0
12	3.41	34	2.2	2.27	22	3.2	2.62	31	2 8	2.97	25	0.4
14	3.80	35	2.4	1.96	18	2.0	3.57	30	4.2	2.62	27	0.8
16	2.69	37	3.4	2.36	23	1.6	3.78	32	1.8	2.65	24	1.6
18	3.59	39	0.8	2.35	19	2.4	3 50	29	2.6	2.71	26	1.2
20				2.43	23	3.0	3.46	31	3.2	2.81	23	1.2
22				1.41	21	3.8				2.88	28	0.8
24	3.52	39	1.0	1.90	21	3.0				2.77	26	0.6
26				1.94	22	2.0				2.46	21	1.0
28	3.28	37	1.2	2.38	22	2.4				2.33	25	2.0
weeks												
6	3.62	36								2.53	27	1.8
8	3.95	41					3.03	27	2.4			
10	3.89	39					3 09	27	2.0			
12												
14												
16												
18												
20	4.23	40	1.6									

	CASE 57			CASE 59			CASE 60			CASE 63		
	Red blood cells	Hemo-globin	Reticulo-cytes	Red blood cells	Hemo-globin	Reticulo-cytes	Red blood cells	Hemo-globin	Reticulo-cytes	Red blood cells	Hemo-globin	Reticulo-cytes
days	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*
0	3.17	42	1.0	2 20	33	3.8	2.03	22	2.4	4.31	38	1.2
2	3.35	44	1.2	2.77	29	3 8	1.98	22	5.6	4.54	39	1.0
4	3.28	44	1.8	3.02	30	3.0	2.20	25	4.0	4.32	35	1.6
6	3.41	45	0.4	2.75	29	2 6	2.19	22	3.8	3.65	32	1.8
8	3.59	45	2.4	2.78	34	2 2	2.21	22	2.2	3.81	37	1.4
10	3.74	48	1.0	3.06	32	1.8	2.42	22	4.6	4.35	33	2.2
12	4 05	46	0.8	3.76	31	1.6	2.20	26	3.8	4.06	38	1.6
14	3 52	50	1.0	3.83	33	2.4	2.42	28	3.2	4 06	39	3.2
16	3.60	50	1.2	3.01	28	3.0	2.74	25	2.0	3.88	37	1.8
18	3.76	53	2.6				2.71	25	3.0	4 00	39	1.6
20	3 66	49	1.0				2.50	26	3.8	4.00	35	1.2
22	3.84	55	1.0				2.25	21	5.8	3.80	38	1.6
24	3.61	57	2 6				2 39	22	3 4	3 86	39	2.8
26												
28	3.61	47	2 0									
weeks												
6	4.12	50	0.6									
8												
10												
12												
14												
16	4.31	60										
18												
20												

	CASE 67			CASE 68			CASE 69			CASE 81		
	Red blood cells	Hemo-globin	Reticulo-cytes	Red blood cells	Hemo-globin	Reticulo-cytes	Red blood cells	Hemo-globin	Reticulo-cytes	Red blood cells	Hemo-globin	Reticulo-cytes
days	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*
0	3 00	37	1.2	2.32	23	3 2	2 48	32	5.2	3.36	40	1.4
2	3.02	38	1.8	2.34	25	3.0	2 53	32	3.4	3.62	44	1.8
4	2.56	34	1.8	2.28	25	2.4	2.76	37	5.0	3.15	35	0.6
6	3.08	36	0.8	2 21	22	0.8	2 70	34	2.6	3.33	37	1.2
8	3 26	35	1.0	2.47	25	1.0	2 57	28	3.2	3.40	42	2.2
10	3.08	37	0.8	2.39	25	1 6	2 65	31	3.6	3.40	41	2.4
12	2.31	31	1.2	2.40	25	3.4	2 60	34	3.6	3.29	39	1.6
14	3.38	36	1.2	2.38	21	2.8	2.44	32	4.2	3.60	40	2.0
16	3.09	34	0.8	2.35	23	2.4	2.42	31	3.2	3 60	37	2.0
18	2.96	37	1.8	2.38	25	2.8	2 64	35	2.8			
20	3 55	41	2.8	2.28	26	2.8	2.73	32	4.2			
22												
24				2.00	25	3.8	2.53	30	4.8			
26												
28				2.94	31	2.4	3.09	35	2.8			
weeks												
6	3.22	33		3.10	30	1.6	3 25	36	3.0			
8												
10												
12												
14												
16												
18												
20												

Second periods. Daily administration 6 grams of ferric ammonium citrate

days																																							
0	4.23	40	1.6	2.38	22	2.4				2.53	27	1.8				3 01	28	3.0	2.39	22	3.4	3.86	39	2.8	3.22	38													
2				2.10	22	6.4				2.98	32	6.4				3.39	34	7.2	2.52	31	9 8	3.97	34	3.2															
4				2.40	28	*10.8*				2.98	29	8.0				3.60	36	6.8	2.74	30	*13.4*	4 44	45	5 4															
6				2.70	31	7 0				3.32	35	*11.8*				3.78	44	*10.8*	3.14	36	6.4	4.51	45	6.8															
8				2.46	25	3.2				3.49	39	7.0				4.10	46	9.0	2.87	32	2.8	4.84	42	5 4	3.11	36													
10				2.74	30	2.6				3.59	38	4.2				4.47	51	8 6	2.99	38	2.2	4.86	45	*7.0*															
12				—	—	—				3 68	43	3.2							—	—	—	4 61	51	4.0															
14	4.81	48	2.8							3.96	44	5.0				4.68	55	5.0				—	—	—	3.73	39	2.0												
16																																							
18										3.87	47	3.8																											
20										—	—	—				4.37	60								3.65	54	2.4												
22	5.12	55	1.2													—	—	—																					
24																																							
26																																							
28	5.02	61	1.6																						4 08	59	1.0												
weeks																																							
6	5.30	68	0.8																						4 39	65													
8	5.31	80																							4 67	72													
Number of worms removed...	1,098			344			313			764			562			1.066			718			103			724			1,234			1,106			40					

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[*] Number of hookworms removed from each patient is given at bottom of table.

trast to this was the positive effect of the subsequent daily administration of 6 grams of ferric ammonium citrate to 5 of these patients. Characteristic reticulocyte responses attended by rapid increases of red blood cell and hemoglobin values appeared in each instance.

It is thus clear that the bone marrow in at least 5 of these patients was in a condition to produce reticulocytes in response to the administration of hematopoietic substances, and therefore could potentially have so responded as a result of the removal of hypothetical inhibitory substances abolished with the hookworm elimination. The negative effect of the removal of the hookworms upon hemoglobin production in cases 13 and 67, in contrast to the striking effect of iron therapy, is graphically portrayed in figure 3. Over periods of 118 and forty-six days, respectively, after the beginning of the hookworm elimination, no

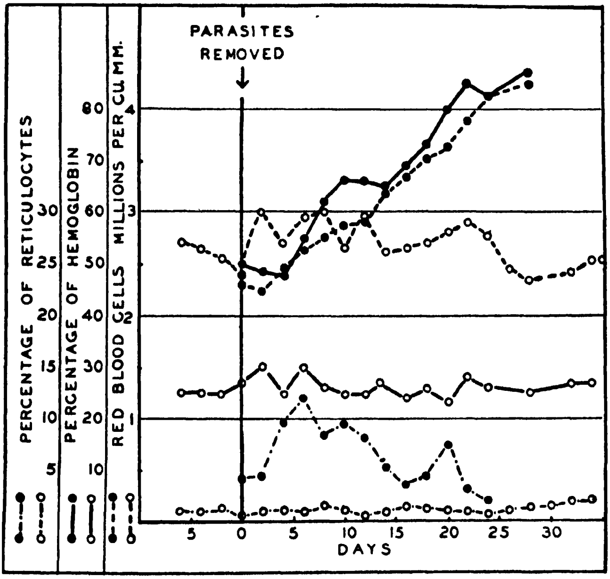

FIG. 2. COMPARISON OF THE EFFECTS UPON HEMATOPOIESIS OF THE REMOVAL OF THE PARASITES IN A PATIENT WITH ANEMIA ASSOCIATED WITH MALARIAL INFECTION (SOLID DOTS), AND IN A PATIENT WITH ANEMIA ASSOCIATED WITH HOOKWORM INFECTION, CASE 52 (OPEN DOTS)

After abolition of the parasites, note the increase of reticulocytes in malaria, and the absence of such effect in hookworm disease. Note also the rapid improvement of the blood values in the anemia of malaria, and the absence of improvement in the anemia of hookworm disease.

significant increase of hemoglobin was noted. These results were further confirmed by the field observations at Cidra.

The effect of hematopoietic substances upon blood production without removal of the hookworms. The second of the two criteria of the possible influence of the parasite in inhibiting bone marrow function was stated to be a determination of the effect of the presence of the parasite upon the production of blood in response to the administration of hematopoietic substances. If the presence of the parasites produced in some way an inhibition of the bone marrow similar to that of other infectious agents, the depressing effect upon the reticulocyte production and increases of red blood cells and hemoglobin should theoretically be detectable. For this reason the response of the hypochromic anemias associated with bacterial infection to the administration of hematopoietic substances is disappointing in contrast to the effect of such substances in similar types of anemia not associated with infection. The inhibitory effect of infection is also occasionally demon-

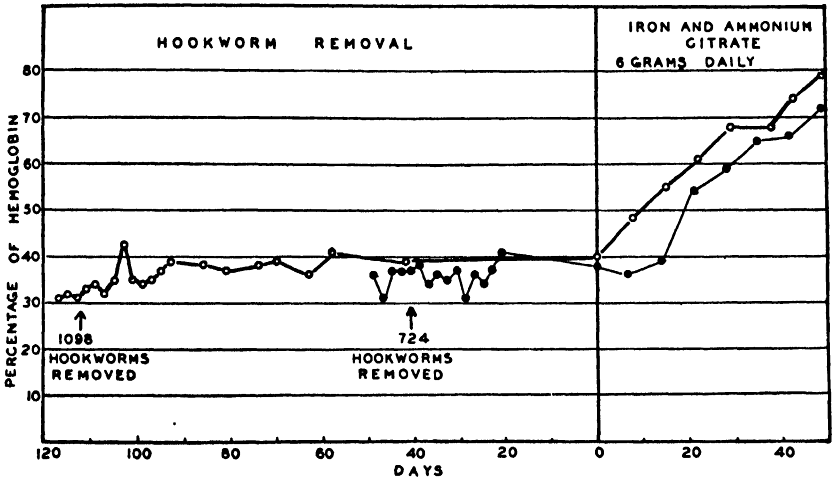

FIG. 3. COMPARISON OF THE EFFECTS UPON HEMOGLOBIN PRODUCTION OF THE REMOVAL OF THE HOOKWORMS AND OF THERAPY WITH IRON

Note the negative effect upon hemoglobin production during a period of one hundred and ten days in case 13 (open dots), and during a period of forty days in case 67 (solid dots), following the removal of the hookworms. Compare the striking subsequent effect on hemoglobin production of the administration of 6 grams of ferric ammonium citrate daily.

plished in increasing knowledge of the causes of sterility or low fertility.

NUTRITION AND REPRODUCTION

by M. H. Friedman and W. A. Turner[3]

At one time or another claims have been made that almost every dietary ingredient is indispensable for normal breeding performance. To some extent this is undoubtedly correct insofar as normal breeding performance is associated with general health. The matter is considerably different if the question is asked, What are the requirements for reproduction above those necessary to maintain a mature animal in good health and to support normal growth in the immature animal? If the discussion is limited to breeding and excludes lactation, the answer is none—with one or two questionable exceptions. Undeniably, there are conditions on deficient pasture and on restricted diet under natural conditions in which breeding performance is below par, but these examples do not afford evidence that the requirements for reproduction are different quantitatively or qualitatively from the requirements for growth and maintenance. These naturally occurring difficulties, moreover, do not incriminate any one element of the diet specifically, because these deficiencies usually involve not one dietary element but several elements at the same time. For example, pasture deficient in phosphorus is deficient in protein also (*494*), and perhaps in still other nutritive ingredients not yet investigated.

For the satisfactory determination of the dietary requirements for reproduction we must rely upon carefully controlled experiments in which the deficiencies are limited to single dietary ingredients, with all other ingredients supplied in ample amounts. There are not many data available from experiments of this character on the larger animals, so that we are forced to be guided by the laboratory data secured from experiments on the rat. In many other kinds of work it is justifiable to assume that results obtained with the rat will be very similar to results obtainable from the larger animals. In nutrition, however, the significant differences between species are numerous enough to prohibit the use of any one species of animal for the determination of the nutritional requirements of other animals. It is therefore necessary to examine the available data critically, realizing their possible shortcomings, and citing those instances in which data from the larger animals are in accord with the results that have been obtained with the rat.

THE RELATION OF PLANE OF NUTRITION TO REPRODUCTION

In the rat serious abnormalities of the sexual function are encountered on a completely balanced ration, even when the food intake is sufficient to maintain a body weight of 200 grams though insufficient to permit any further gain in weight (*344*). In terms of energy intake, a decrease of not more than 25 to 30 percent below what the rat consumes on a self-feeding regime will affect reproduction adversely

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(*344, 738*). With underfeeding severe enough to cause a marked retardation in the growth of the immature animal, or a loss of weight in the mature animal, the estrus cycle stops completely, ovulation fails entirely, the testes show atrophy and a loss of motile sperm, and the male loses sex interest and vigor (*38, 344, 738, 748, 761*). The female rat may show these abnormalities in as little as 2 days (*748*). The guinea pig is almost equally susceptible to reduced food intake (*692, 893*). Although the breeding performance of Nigerian (Africa) cattle afford an example of the effects of restricted energy intake (*22*), there has been no extensive investigation of this question in farm animals as a whole.

This also applies to studies on human beings, all of the data available being complicated by specific dietary deficiencies in addition to energy deficiency. For example, the lowered fertility of both the human and animal populations in central Europe following the World War may have been due to underfeeding, but it must be remembered that the diet in these countries was severely restricted with respect to protein.

On the other hand, almost all nutritional deficiencies in animals are complicated by underfeeding. Any diet deficient in one or more ingredients is apparently less palatable to the animal than a well-balanced ration, so that food intake on the deficient ration is much below normal. If the missing items are added to the deficient ration, the food intake increases and a gain in weight follows.

Because of this situation, it is almost impossible to arrange a satisfactory laboratory experiment that will show the effects of the deficiency of a single dietary ingredient without encountering at the same time the unavoidable effects of partial starvation. Practically every experiment on the rat or on larger animals reported in the literature suffers from this complication. Almost without exception, animals on the deficient diets eat less than the control animals on the complete ration. Obviously the only fair comparisons in such situations would be with control animals limited to the same energy intake as the experimental animals. To put the situation in another way, it is necessary for the experimental animals to eat the ration if the effects of this ration are to be studied. From a scientific standpoint, it does not matter whether an animal starves to death by refusing to eat a diet low in phosphorus or by refusing to eat a diet low in iron. In either event death would be attributable to starvation and not to a specific deficiency of phosphorus or iron.

In view of the dramatic effects of undernutrition on reproduction it is something of a paradox that a high plane of nutrition is suspected of leading to sterility. Yet those who accept as fact that what is called "high condition" leads to sterility also accept without question the practice of flushing sheep. These two ideas do not fit well together unless we can accept the proposition that a high plane of nutrition for a short time leads to increased fertility, but over a longer period of time leads to sterility.

The practice of flushing sheep needs no defense by this time. Well-controlled experiments (*212, 262, 750, 752, 858*) in several countries have amply demonstrated that flushing results in a larger lamb crop even though it will not bring about heat at an earlier date. For

TABLE 3

Effect upon hematopoiesis of daily addition of 6 grams of ferric ammonium citrate (second periods) to basal diet without removal of hookworms

	LIVER EXTRACT NO. 343 N.N.R. DERIVED FROM 10 GRAMS OF LIVER INTRAMUSCULARLY									NO THERAPY			FERRIC AMMONIUM CITRATE, 100 MGM. INTRAMUSCULARLY			NO THERAPY			FERRIC AMMONIUM CITRATE, 100 MGM. INTRAMUSCULARLY						NO THERAPY					
	Case 5			Case 9			Case 11			Case 16			Case 23			Case 47			Case 56			Case 66			Case 69			Case 74		
	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes
First periods. Either no therapy or daily administration of various substances as indicated																														
days	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*
0	3.63	50	1.4	2.36	29	2.7	2.29	37	1.9				2.76	28	2.6				3.53	36	1.4	3.15	34	1.6	2.88	33	1.0			
2	3.79	47	1.4	2.89	33	2.6	2.29	22	1.1				2.46	26	2.8				3.50	38	2.4	2.65	33	1.6	3.02	32	1.4			
4	3.64	45	1.6	2.89	33	0.4	2.42	23	2.4				2.39	24	*6.0*				3.55	38	2.0	2.73	35	2.1	3.12	35	1.0			
6	3.45	45	3.0	2.74	34	1.8	2.53	21	3.8				2.37	25	4.2				3.39	41	*3.4*	3.11	36	*6.6*	—	—	—			
8	3.85	46	2.0	2.44	30	1.2	1.96	20	3.4				2.53	25	4.8				3.34	40	3.2	3.14	38	4.6						
10	3.29	46	1.8	3.17	34	4.0	1.91	21	1.4				—	—	—				—	—	—	—	—	—						
12	—	—	—	2.98	32	2.4	—	—	—																					
Second periods. Daily oral administration of 6 grams of ferric ammonium citrate																														
0	3.29	46	1.8	2.98	32	2.4	1.91	21	1.4	2.42	22	1.8	2.53	25	4.8	1.93	26	9.0	3.34	40	3.2	3.14	38	4.6	3.12	35	1.0	3.54	34	1.6
2	3.38	44	3.1	3.40	37	2.6	2.16	25	5.8				2.76	27	5.6				3.73	41	5.2	3.53	43	3.6	3.00	37	1.2	3.48	35	1.8
4	3.51	44	4.6	3.03	38	8.0	2.47	25	*13.8*				2.59	30	3.6	2.18	34	13.6	3.81	41	4.2	3.52	44	5.4				3.20	37	2.0
6	3.42	48	6.0	3.00	39	*9.0*	2.58	30	10.8	2.96	34	11.0	2.93	35	4.6				3.86	45	6.6	3.84	46	*6.2*	2.94	41	8.0	3.36	38	2.2
8	3.46	42	4.6	3.24	41	3.6	2.95	33	7.8	3.47	41	*16.4*	2.84	35	4.2	3.11	47	*23.0*	3.76	49	2.9	3.69	44	4.6	3.13	42	*7.2*	3.68	40	2.4
10	3.36	48	4.2	3.14	47	4.6	3.50	40	2.0	3.20	44	6.4	2.94	37	2.4				3.97	50	3.2	3.63	51	1.4	3.80	45	5.6	3.65	41	2.2
12	3.43	54	7.4	3.32	51	2.4	3.65	44		3.65	50	11.8	3.18	37	2.0	3.14	52	5.8	3.87	54	2.0	3.87	50	3.2				3.33	43	1.2
14	3.47	53	7.2	3.93	53	2.0							3.02	38	0.1				4.47	59	*4.0*	3.48	50	1.8	3.56	44	4.4	3.73	44	1.2
16	3.60	58	*9.8*	3.96	56	2.2	3.87	47	3.0	3.56	52	5.4	3.26	41	1.8	3.09	59	3.2	4.17	61	1.6	3.71	53	3.4	3.76	47	3.6	3.83	50	0.4

18	3.76	58	5.6	4.12	58	2.0	3.63	48	2.2				3.15	41	2.8										4.09	48	2.2	3.71	50	0.8
20	3.84	58	3.0	4.11	60	1.8	3.92	53	2.4				3.39	46	1.8				4.20	62	0.8							3.73	52	1.4
22	3.99	62	5.2				3.79	50	4.8				3.28	44	2.4	3.23	53	1.8										3.68	54	1.0
24	4.04	64	1.8				3.90	55	3.2				3.39	48	2.4	—	—	—	4.19	65		3.68	58	2.6				3.47	50	1.4
26	4.15	63	4.6				4.14	62	2.0				3.46	45	2.2										3.77	56		4.02	59	0.8
28	4.30	66	2.2	4.29	64	0.1	3.98	57	2.4	3.37	66	3.6	3.37	53	3.6				4.61	68	0.8	4.00	64	3.0				3.40	56	0.8
weeks													—	—	—													—	—	—
6	4.30	71	4.4	4.39	70	1.4	4.24	64	2.0	3.27	65	2.4							4.83	72	1.0	4.15	76	3.2	4.11	67	1.2			
8	—	—	—	4.73	83		4.44	70	2.2	4.07	71	1.0							—	—	—	4.41	80	0.8	4.73	68	0.4			
10				—	—	—	4.11	68		3.79	73											—	—	—	4.45	73				
12							4.08	57		3.43	74														4.21	70	1.0			
14							—	—	—	3.55	69														4.61	74	0.6			
Ova per cgm. stool	88			201			119			44			48			307			Positive			723 worms*			30			Positive		

---

* Number of hookworms removed after termination of the observation.

TABLE 4

Effect upon hemoglobin production of daily administration of 6-grams of ferric ammonium citrate

CASE NUMBER	HEMOGLOBIN PERCENTAGES AT 10-DAY INTERVALS						
	0 days	10 days	20 days	30 days	40 days	50 days	60 days
Hookworms not removed							
4	35	38	48	55	63	68	
5	44	48	58	68	71		
7	44	55	64	70	76	83	85
8	34	40	57	63	72	75	83
11	21	40	52	57	61	66	70
16	22	44	54	64	68	70	72
23	25	37	46	53			
24	44	57	66	76			
34	43	53					
37	18	34	41	50	49	52	
38	42	49	57	64	72	77	
39	17	20	26	32	35	41	44
41	36	42	52	56			
42	25	33	43	55	61	67	69
43	35	40	42				
46	41	50	59	65	69	71	73
47	26	47	56				
48	30	40					
56	40	50	62	68	72		
66	40	51	58	62	70	78	80
69	35	45	52	59	64	68	70
71	41	50	55				
72	22	30	41	50			
73	36	50					
74	34	41	51	56			
76	45	58	63				
80	41	52	66	62	70		
Averages....	34	44	53	59	65	68	72
Hookworms previously removed							
13	40	50	59	68	70	80	
52	27	38	47				
59	33	47	59				
60	22	37					
63	36	48					
67	38	37	52	60	66	71	
Averages....	33	43	53	64	68	76	

citrate fortunately bring additional evidence to bear on the question. In 5 patients whose hookworm burden was removed, during the first thirty days the average gain in hemoglobin (35 per cent) was slightly less than the average gain in hemoglobin (38 per cent) of 7 patients who had received no treatment for the parasites. Each group gained 44 per cent of hemoglobin during the first sixty days of therapy with iron. The hemoglobin percentages for each patient were calculated for ten-day intervals as described above, and, together with the averages, are given in table 9. The average hemoglobin values for each group are also plotted at ten-day intervals in figure 5. It is reasonable to conclude, therefore, that at least no evidence for a significant inhib-

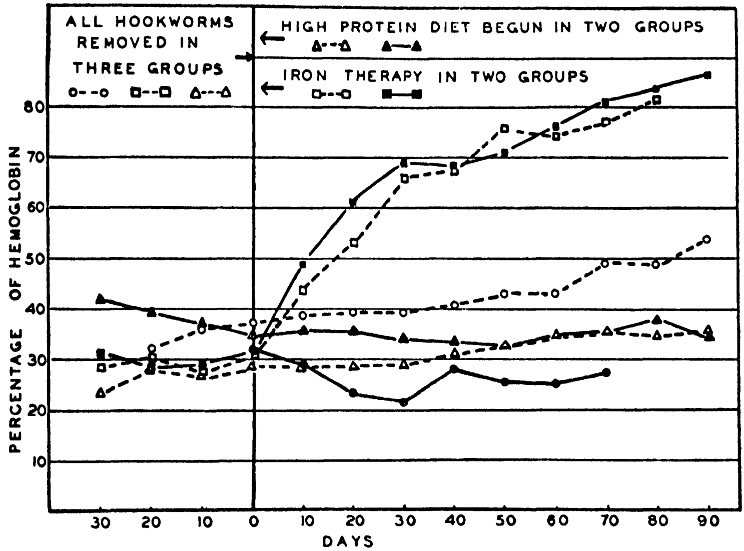

FIG. 5. COMPARISON OF THE EFFECTS OF REMOVAL OF THE PARASITES, OF IMPROVED DIETS, AND OF THERAPY WITH IRON. RESULTS OF OBSERVATIONS IN THE FIELD AT CIDRA ON THE AVERAGE HEMOGLOBIN PRODUCTION IN 6 GROUPS OF PATIENTS

The 32 patients were divided into two main groups, A and B, with three subgroups each, I, II and III respectively. The 16 patients of group A (open symbols) had the hookworms removed before the beginning of the experimental period (vertical line in figure). The 16 patients of group B (solid symbols) received no treatment for hookworms. The patients of groups A-I and B-I (circles) had no change in diet. The patients of groups A-II and B-II (triangles) were given as an addition to their usual diet 300 grams of meat and 1500 cc. of milk daily. The patients of groups A-III and B-III (squares) had no change in diet, but received daily 6 grams of ferric ammonium citrate. Note the positive effects of iron in groups A-III and B-III in contrast to the negative or slight effects of removal of the hookworms or improvement of the diet.

itory effect of the parasites upon the bone marrow was demonstrable by our observations.

b. The effect of blood loss. It is clear from the experiments of Wells (28) that at least in the dog considerable amounts of blood are removed by the hookworm. He estimated that in twenty-four hours 100 hookworms removed 84 cc. of blood. Although Smillie (29) states that a large amount of blood is never seen in the intestinal tract of *Necator*, it is reasonable to suppose that some blood loss does occur as the result of infection with this as with other species. According to Bass (30) tests of the feces for occult blood are nearly always positive, even in mild or moderate infections. In our experience hemorrhagic spots were observed at the point of attachment of the worms in the intestinal tract of autopsied patients, and the worms would ingest red blood cells after being placed in saline solution.

The anemia of acute blood loss is a condition from which the individual in a normal state of nutrition promptly recovers, and the evidences of increased bone marrow activity are ordinarily shown in the peripheral blood by the appearance of augmented numbers of reticulocytes and occasionally of nucleated elements. At the same time, the red blood cell and hemoglobin values progressively increase toward normal. On the other hand, both animal experiments (31) and clinical observations make it clear that if the blood loss is sufficiently chronic, especially in the presence of dietary deficiency, hypochromic anemia without evidence of active blood regeneration will result when the available hematopoietic substances have been depleted to a certain level. The blood picture, under these circumstances, no longer shows evidence of increased bone marrow activity; the concentration of hemoglobin in the red cells becomes much reduced, and typical hypochromic anemia results.

McMaster and Haessler (32) showed that in rabbits in which anemia was induced by repeated bleeding, the blood was more quickly made up if the animals were given subcutaneous injections of hemoglobin. Miller and Rhoads (33) have recently shown that rabbits maintained at a hemoglobin level of approximately 50 per cent of normal by daily bleeding, will produce practically twice as much hemoglobin in a given length of time when the blood removed is laked and the hemoglobin reinjected as when the blood is discarded. Whipple

TABLE 5

Estimate of the number of times per month food of type specified was eaten by patients

CASE NUMBER	MEAT	MILK	EGGS	FISH	CEREALS	GREEN VEGETABLES	FRUIT	BUTTER
2	2	8	4	4	0	4	4	8
4	8	60	30	8	30	8	4	
5	8	8	8	12	8	12	30	
6	60	60*	30	2	0	12	60	30
7	8	30	30	2	30	60	30	30
8	3	1*	1	12	0	2	30	12
9	12	90	8	8	1	30	8	8
11	0	0	0	30	0	8	90	0
13	12	60	8	12	0	8	2	0
15	0	0	4	4	0	0	30	
16	0	30*	30	4	0	12	60	30
23	2	4	8	8	1	3	12	8
24	20	60*	60	20	1	30	60	30
25	2	60*	12	30	0	12	8	8
26	8	90	12	12	0	3	30	4
29								
30	4	30*	12	30	0	3	12	
32	0	0	8	8	0	30	30	
33	4	0	12	12	0	4	12	
34	0	0	0	30	0	12	90	0
35	8	30*	30	2	0	0	60	0
36	1	0	8	20	0	4	12	
37	20	30	30	8	0	12	12	
40	8	0	4	1	0	4	20	0
41	4	60	2	8	0	12	60	4
43	3	60	2	8	0	1	2	2
44	4	0	30	8	0	8	12	
45	0	20	1	8	0	2	4	
46	0	0	4	0	0	2	8	
48	1	4	4	30	0	8	8	4
49	4	30	0	8	0	3	30	0
50	4	20	4	4	0	2	2	0
51	8	30	4	8	1	4	20	4
52	3	30	12	20	0	3	30	0
53	8	0	8	20	4	4	30	
54	4	20	8	4	0	4	4	
56	12	30	0	1	0	60	30	0
57	8	30	4	0	0	8	8	4
58	6	30	30	12	12	30	30	30
59	12	30	4	16	4	30	20	0
61	12	30	2	24	2	60	40	2
62	12	0	0	8	0	12	2	30

TABLE 5—*Concluded*

CASE NUMBER	MEAT	MILK	EGGS	FISH	CEREALS	GREEN VEGETABLES	FRUIT	BUTTER
64	8	20	4	12	0	30	4	0
65	4	60*	30	30	0	12	90	0
66	12	60	8	16	3	8	24	4
67	12	4*	4	30	0	8	8	
68	0	30*	8	30	0	8	30	1
69	0	16*	12	30	0	12	6	0
71	20	60	0	8	4	30	30	0
72	4	4	8	30	0	8	8	
74	4	30	2	20	0	30	30	20
75	8	30	12	0	0	30	20	30
79	8	20	4	4	0	30	30	0
80	12	30	30	20	0	8	60	
81	0	0	0	4	0	0	4	
82	32	30	30	16	0	30	4	2

* Milk other than in coffee.

and Robscheit-Robbins (34) have demonstrated that in dogs with severe anemia from blood loss there is an 80 to 90 per cent utilization of hemoglobin given intravenously or intraperitoneally. The present observations demonstrate that human red blood cells also contain hematopoietic substances. Into each of 2 patients, cases 20 and 36, 30 cc. of washed, packed human red blood cells were injected intramuscularly daily for six days. The hematological data are shown in table 6. Although the results of the injection of these relatively small amounts of red blood cells, assuming even a 90 per cent utilization, could not be expected to be striking, it is interesting to note that in case 20 a gain of 1.97 red blood cells per cubic millimeter and 9 per cent of hemoglobin resulted within a period of sixteen days. In case 36 the gain of red blood cells and hemoglobin was insignificant, but a definite increase of reticulocytes appeared and reached a maximum of 12.6 per cent on the eighteenth day after the beginning of the injections. Similarly, in case 20 a reticulocyte peak of 6.8 per cent was attained on the twelfth day. No hematopoietic effects were observed from the injection of cell-free plasma. These results are considered to add to the evidence from other types of observations (32) (33) (34) that blood loss is important in the production of hypochromic anemia, not only as a result of the actual blood shed, but also as a result of the deprivation of the body of hematopoietic factors contained in the lost red blood cells. Whatever blood loss is then due to the hookworm is clearly a potential contributory factor to the development of the hypochromic anemia through both of these mechanisms.

2. *The relation of factors other than the hookworm to the anemia.* The work of many authors, especially that of Meulengracht (35), Witts (36), and Heath (37), suggests that hypochromic anemia in the temperate zone is due mainly to a lack of available iron in the body. The failure of the bone marrow to produce adequate amounts of hemoglobin and red blood cells is apparently at the bottom of the problem, and results directly or indirectly from an inability of the bone marrow to function normally in the absence of sufficient iron and probably of other hematopoietic substances. In hypochromic anemia, not associated with infection, the mechanisms by which the failure of hematopoiesis is brought about are various and may be combined in the same patient. The available iron in the body may be depleted by blood loss or the iron intake may be insufficient either because of dietary defect or because the absorption of iron from the intestinal tract is inadequate. In the latter case gastric anacidity (38) and conditions producing diarrhoea (39) probably play an important rôle. Although the ability of the hookworm to cause blood loss is clear, the failure of correlations of the degree of anemia with the number of parasites harbored by different individuals, clearly suggests the influence of additional factors. Furthermore, those communities in which hypochromic anemia associated with hookworm infestation is common, also furnish examples of similar types of anemia in individuals who have never had hookworm disease. Since, as has been demonstrated, the hypochromic anemia of hookworm disease has no morphological or physiological characteristics which distinguish it from hypochromic anemias without hookworm infestation, it is logical to look for the possible influence of factors common in producing other types of hypochromic anemia.

a. The relation of dietary defects to the anemia. Smillie (5) has suggested the importance of malnutrition in augmenting the anemia of hookworm disease in Brazil. Smillie and Augustine (23) thought

TABLE 6

Comparison of effects upon hematopoiesis of administration of various extracts of liver, of washed erythrocytes and of ferric ammonium citrate to patients upon basal diets without removal of hookworms

First periods. Daily administration of various substances as indicated below

	LIVER EXTRACT E-29 DERIVED FROM 340 GRAMS OF LIVER (CONCENTRATED AQUEOUS EXTRACT)												LIVER EXTRACT NO. 55, DERIVED FROM 400 GRAMS OF LIVER WITHOUT ADDED IRON (FRACTION INSOLUBLE IN 70 PER CENT ALCOHOL)									LIVER EXTRACT NO. 343, N.N.R. DERIVED FROM 300 GRAMS OF LIVER (FRACTION SOLUBLE IN 70; INSOLUBLE IN 95 PER CENT ALCOHOL)						WASHED ERYTHROCYTES FROM 30 CC. NORMAL BLOOD INTRAMUSCULARLY DURING FIRST 6 DAYS					
	Case 1			Case 8			Case 26*			Case 64			Case 22			Case 51			Case 36b			Case 27			Case 61			Case 20			Case 36a**		
	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes
days	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*
0	3.91	35	1.6	2.53	32	5.2	1.07	10	4.4	3.57	44	5.2	2.11	24	2.4	3.17	37	2.2	2.06	20	5.4	2.09	22	6.4	2.02	21	2.4	2.26	30	2.8	1.41	17	3.0
2	4.02	34	1.8	2.46	35	4.0	1.16	9	3.0	3.67	42	4.0	2.32	26	2.2	3.07	34	—	1.95	20	4.2	2.24	23	4.0	2.12	18	0.8	2.97	26	2.0	1.43	16	2.4
4	3.78	40	1.4	2.66	34	5.4	1.01	9	2.6	3.03	41	5.8	2.50	23	3.6	2.84	35	2.0	1.73	20	6.6	2.19	24	1.6	1.89	19	1.8	3.27	28	2.6	1.63	15	3.6
6	3.68	38	3.0	2.86	40	*13.8*	1.13	13	*13.8*	3.60	42	*8.0*	2.15	23	2.4	3.13	33	1.0	2.47	22	9.0	2.14	24	4.8	1.81	21	2.6	3.49	29	2.6	1.42	15	2.8
8	3.41	37	*5.0*	3.14	42	12.6	1.34	18	9.8	3.54	44	5.0	2.26	20	2.8	3.17	34	4.2	2.49	26	*13.4*	2.26	26	3.8	1.94	20	1.2	2.86	30	5.0	1.21	15	3.4
10	4.06	42	2.8	3.30	43	10.6	1.31	19	7.4	3.65	43	6.0	2.38	19	2.8	2.86	33	*5.2*	2.38	26	8.4	2.53	25	3.8	1.86	20	4.8	3.28	31	6.6	1.08	13	5.0
12	4.00	39	3.4	3.47	50	7.6										3.27	37	2.8				2.60	28	2.6				4.11	35	*6.8*	1.09	14	10.0
14				3.59	49	4.8																						4.22	36	3.6	1.33	14	8.8
16	3.86	43	1.0	4.02	58	5.0																						4.23	39	5.2	1.38	16	8.8
18																															1.99	22	*12.6*
20																															1.54	20	4.6
22	4.04	47	2.2																														
24																																	
26																																	
28	4.05	53	3.4																														
30																																	
32																																	
34	4.08	51																															

* Received extract from only 240 grams of liver daily.

** Periods preceding other observations shown above on same case (36b)

Second periods. Daily administration of various substances as indicated below

	FERRIC AMMONIUM CITRATE, 6 GRAMS						FERRIC AMMONIUM CITRATE, 4 GRAMS			FERRIC AMMONIUM CITRATE, 6 GRAMS		
0	4.08	51		4.02	58	5.0	1.31	19	7.4	3.65	43	6.0
2				3.91	59	3.0	1.36	26	14.0	3.82	47	3.8
4				3.80	60	*6.0*	2.03	27	*17.0*	3.77	48	6.6
6				3.70	66	4.4	2.21	31	10.0	3.33	47	5.8
8	3.73	56		4.00	61	3.2	2.23	28	13.4	3.93	52	*8.4*
10				4.23	69	2.2	2.80	35	7 8	3.74	57	3.8
12							3.08	39	14.2	—	—	—
14	4.78	60					3.19	40	7 6			
16							3.26	44	6.8			
18							3.21	46	4.4			
20							3.43	45	3 8			
22	3.63	57		4.43	72	1.0	3.51	48	4 8			
24							3.21	46	4.8			
26							3.58	51	2.0			
28	3.78	57										
30	—	—	—									
32												
34				4.74	75		4 60	67	1.4			
weeks												
6												
8				3.81	72	2 4	4 41	63	0 6			
10				4.38	76		4.00	65	1.4			
Ova per cgm. stool....	Positive			357			494			118		

	FERRIC AMMONIUM CITRATE, 2 GRAMS						FERRIC AMMONIUM CITRATE, 6 GRAMS		
0	2 38	19	2.8	3 27	37	2.8	2 33	26	8 4
2	2 30	22	2.8	3.48	35	2 8	2 86	31	8 2
4	2 55	26	5 2	3 58	38	6 2	2 80	36	*14 0*
6	2 65	29	*10 0*	3 77	36	8 0	2 93	44	9 6
8	2.98	32	10 0	3 50	38	*8 2*	2 83	49	8.8
10	3 38	39	9 6	4 15	40	7 0	2 94	52	8 0
12	3.31	37	8 6	4 08	46	6 0	3 04	49	4.4
14	3 46	42	8.2	3.89	43	5 4	3 60	60	4 8
16	3.36	47	8.0	3.54	43	3 8	3.45	56	4 0
18	3 67	52		3 47	43	4 0			
20	3 49	58	7 0	*3 61*	*44*	*4 6*			
22	3 61	57	6 4	*Fe. Am. Citr. 6 gm.*					
24	3 96	59	3.4	3 91	53	8.4	4 04	65	2 0
26	3 91	58		4 06	58	*10.4*			
28	4 42	66	1 2	4 33	57	6.6			
30	—	—	—	4 14	60	6 0	4.41	73	1.2
32									
34									
weeks									
6							4.13	79	
8							—	—	—
10									
Ova per cgm. stool....	Positive			250			130		

	LIVER EXTRACT E-29 DERIVED FROM 340 GRAMS OF LIVER						FERRIC AMMONIUM CITRATE 50 MGM. INTRAMUSCULARLY FIRST 6 DAYS			FERRIC AMMONIUM CITRATE 100 MGM. INTRAMUSCULARLY FIRST 5 DAYS		
0	2 60	28	2.6	1.86	20	4.8	4.32	39	5.2	1.54	20	4.6
2	2 63	27	3.2	2 12	23	4.4	4.28	34	5.4	1.61	20	5.4
4	2.87	28	7.6	2 22	20	3.0	4.32	34	4.8	1.74	19	3.8
6	2.83	35	9 8	2.10	23	3 6	3 72	37	5.2	1.43	19	6.6
8	3 14	38	10 0	2.59	27	*6.8*	4.22	39	3.6	1.60	23	*7.0*
10	3 53	44	*11 0*	2 79	28	3.8	4.33	39	2 6	1.96	21	5.2
12	3 38	42	5 0	2.74	29	5.8	4.24	40	2.2	2.06	20	5.4
14	3.30	41	5.2	*2 98*	*34*	*3 8*	—	—	—	1.95	20	4.2
16	3 47	45	5.2	*Fe. Am. Citr. 6 gm.*						—	—	—
18	3 61	45	8.4	3.52	40	*12 6*						
20	3.88	50	6 8	3 46	46	10.4						
22	3 83	49	5 2	3.39	47	4 8						
24	3 89	52	4 4	4 03	51	4.0						
26	3 92	52	3 6	4.14	55	3 6						
28	3.96	54	2.6	4 02	57	1.8						
30												
32												
34	3.33	63	2.2	3 89	65							
weeks												
6	4 12	72		4 14	69	0 2						
8	4 85	78	1.0	4.60	70	1.4						
10	5 20	78	0.6	—	—	—						
Ova per cgm. stool....	286			101			4			130		

that with ample food there could be a heavy hookworm burden without anemia. Schapiro (6) noted differences between the worm burden and degree of anemia in the population of Panama, which he attributed to differences in diet. An investigation was made of the individual diets of 56 of the 83 patients presented in this report selected entirely at random. The method of taking the diet histories included questioning the patient as to his particular likes and dislikes in food, and whether his diet differed in any way from that of other members of his family. He was asked to describe an average daily menu. In addition, he was questioned as to the number of times per day, week or month that he ate specific foods common in Puerto Rican dietaries. Although the taking of a dietary history is admittedly a difficult procedure in which the quality of the patient's answers may be as im-

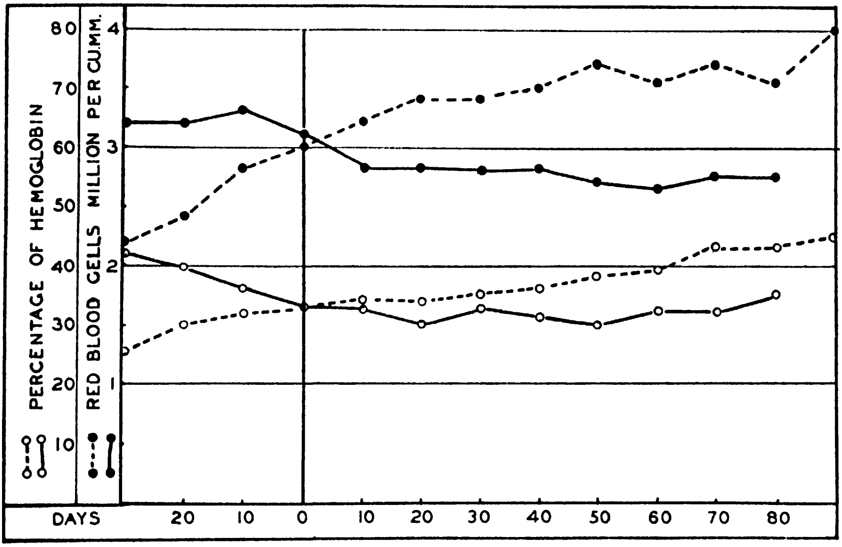

FIG. 6. EFFECT OF THE REMOVAL OF THE PARASITES UPON RED BLOOD CELL AND HEMOGLOBIN PRODUCTION IN PATIENTS NOT RECEIVING IRON

The average values of the red blood cells of the 11 patients of groups A-I and A-II (solid dots and broken line), whose hookworms were removed, should be compared with the average values of the red blood cells of the 9 patients of groups B-I and B-II (solid dots and line), whose hookworm burden was not removed. The average hemoglobin values of the same groups of patients are shown in the curves with open dots. The differences in the trend of the curves represent differences induced by removal of the parasites. Note that the effect of removal of the parasites upon red blood cell production is greater than upon hemoglobin production.

portant as his quantitative statements in indicating the amount and type of food taken, the monotony of the fare of the Puerto Rican peasants aided the investigation by limiting the possibilities. In general, the diets were very similar and could well be described in the words of Suárez (40):

> The Porto Rican peasant breakfasts on coffee and a small loaf of pale white bread. His lunch consists of a large quantity of polished rice and kidney beans with scanty vegetables. His dinner is a stew of rice, chick-peas, pumpkin, and perhaps one or two tropical vegetables such as yautiá. Once or twice a week he has the great good fortune to eat some salted codfish. This diet has been taken without variation since childhood. Many of these individuals have never had meat, eggs, or milk.

In table 5 an attempt has been made to present in a roughly quantitative manner the individual consumption of those articles of diet which might be considered to be important from the standpoint of sources of complete proteins, vitamins, and mineral elements including iron. Thus, the table does not represent the chief sources of energy of the Puerto Rican diet mentioned by Suárez, but merely those foodstuffs considered to be of especial nutritional significance. The figures represent as nearly as could be determined the average number of times, without reference to quantity consumed, that the particular type of food mentioned was eaten during a period of one month. It is to be clearly understood that the figures are estimates and are necessarily of only approximate accuracy. From an inspection of the data, however, it is at once apparent that the consumption of meat, milk, eggs, whole grain cereals, and butter was extremely low in this group of patients. Milk was utilized mainly in small amounts in coffee unless indicated in the table by an asterisk. Fresh fish was rarely used, and the values in the table refer almost entirely to salt codfish. Green vegetables, such as lettuce, chard, string beans, chayote, okra, and other Puerto Rican products were largely employed in garnishing the main staples, rice and beans. In a land in which citrous fruits, pineapples, and bananas are plentiful, it is surprising to see how small the consumption of these articles appeared to be.

The explanation is largely an economic one, for the majority of the population affected by hookworm anemia is of the peasant class, serving as laborers on plantations for the raising of such "gold crops" as sugar, coffee, and tobacco. The concentration of the living quarters of the laborers makes small truck farming difficult, or it may even be expressly forbidden by the policy of the company. The wage scale is low, and the dependents are often numerous. Several of the patients in this group depended for their food entirely upon the charity of others. Poverty, local customs, and lack of refrigeration diminish to a minimum the consumption of meat and dairy products, and result in the widespread use of dry beans and peas, polished rice, and salt codfish in a land seemingly bountifully endowed by nature.

It has been clearly shown by many workers (35) (36) that iron in a variety of forms is of dominant importance in the therapy of hypochromic anemia. Since Heath, Strauss, and Castle (37) have shown that in hypochromic anemia, iron administered parenterally reappears more or less quantitatively in the resultant increased hemoglobin in the blood stream, the view that iron therapy acts more or less directly by eliminating an iron deficit in the body is highly credible. For this reason it is logical to suppose that a lack of iron in the diet may lead to a deficiency of hemoglobin in the red blood cells. According to the figures given by Sherman (41) the percentage iron content of the main articles of these Puerto Rican diets may be assumed to be somewhat as follows: dry kidney beans, 0.007; dry peas, 0.0057; white bread, 0.0009; fruit and tropical vegetables less than 0.001; codfish at least 0.0011. Lean meat and eggs each contain 0.003 per cent of iron, but the other articles itemized in table 5, such as milk, cereal, green vegetables, and butter, all contain less than 0.001 per cent of iron. It is thus apparent that, because of the addition of peas and beans, there exists in these diets a reasonably good source of iron when compared with the figures, for example, for lean meat and eggs. According to Robscheit-Robbins (42) certain foodstuffs, especially glandular organs, such as liver, kidney and chicken gizzard, and such foods as eggs, apricots, and, to a lesser extent, lean meat, are of importance in producing hemoglobin in the dog. It is clear that these articles were present to only a small degree in Puerto Rican diets.

Based on the available knowledge, the diets seem possibly to have contained in the peas and beans good sources of iron, although they lacked decidedly the food substances that by reason of other properties may be of metabolic importance in the production of hemoglobin. In the light of these facts, it is certainly not possible to arrive at any definite conclusion concerning the exact nature of the dietary defect related to the anemia. It is clear, however, that the supply of animal protein, minerals, and possibly of certain vitamins was far from optimal in these diets. Cook (43) states that 50 per cent of the energy value of the foods imported in Puerto Rico is represented by rice and beans. He showed that prepared rice and beans, as served in a Puerto Rican restaurant, were inadequate for growth in rats unless supplemented with cod liver oil. Certain common Puerto Rican vegetables, such as plátano and yautiá, were of some assistance in promoting growth when added to the rice and beans. Cook and Rivera (44) found the calcium intake to be low in the Puerto Rican diets. Although the exact relation of dietary constituents to anemia is still uncertain, the clinical impression is that many cases of hypochromic anemia, not associated with hookworm infestation, are observed in the temperate zone, in which diets far less impressively defective than these appear to be of etiological significance. On the other hand, the appearance of anemia in certain individuals is incomprehensible except on the assumption that changes in the assimilative capacity of the intestinal tract render such individuals different from other members of the community. In the light of the observations of McCarrison (45) and others, it is probable that defective nutrition also exercises an effect indirectly, by promoting changes in the function of the gastrointestinal tract. Such changes may then secondarily lead to difficulty with the assimilation of elements directly essential to hematopoiesis.

b. The relation of gastrointestinal pathology to the anemia. The concept of deficiency disease "conditioned" by gastrointestinal defects has been elaborated, using as an illustration Addisonian pernicious anemia (46). The apparent relation of altered gastrointestinal function to hypochromic anemia has been emphasized by the work of Mettier and Minot (38), Keefer (39) (47), and others (48) (49). Dysphagia, gastric anacidity, and diarrhoea have been shown to occur in many cases of hypochromic anemia and to have a probable etiological relationship by causing the individual to limit or modify his diet or by preventing the proper assimilation of the elements necessary for hematopoiesis. Ashford and his coworkers (8) stated that in hookworm disease the tongue was at times partially denuded of epithelium and was raw and red. They noted that a severe stomatitis was sometimes present, even involving the buccal mucous membranes. Pain and tenderness in the epigastrium were found to be the most constant and clearly marked of any of the symptoms referable to the digestive tract. General abdominal tenderness was occasionally present. In their experience constipation was the rule, but in the advanced stages of the disease this alternated with diarrhoea.

The factors of glossitis, gastric anacidity, and diarrhoea were accordingly given special attention in the analysis of the causes of this type of hypochromic anemia. In table 1 are shown the results of these investigations. Of 47 patients adequately studied, 8 either gave a history of or showed lingual changes. This is of importance because of the common association of loss of the papillae of the tongue in hypochromic anemia and in other deficiency diseases (48) (50). The gastric response to the administration of 50 cc. of 7 per cent alcohol and to the subcutaneous injection of 0.5 mgm. of histamine phosphate was determined in 54 patients, usually once, either before treatment or in the early stages of effective therapy. The pepsin content of the material secreted in response to histamine was estimated by the use of Mett's tubes in samples of gastric contents brought, if necessary, to pH 2 with hydrochloric acid. Of the 54 patients examined, free hydrochloric acid was secreted in response to the alcohol by 30, and only after the injection of histamine by 11; in 13 patients there was no secretion of free hydrochloric acid. Peptic activity was observed in all but 7 patients, of whom only one was able to secrete hydrochloric acid. That gastric anacidity was found in only 24 per cent of the patients with hookworm disease and anemia indicates that it is by no means as common as in idiopathic hypochromic anemia (20) (51). However, since Mettier and Minot (38) were able to demonstrate greater effects with iron salts administered to patients with idiopathic hypochromic anemia when the upper intestinal contents were kept relatively acid than when maintained near the neutral point, the etiological significance of achlorhydria in relation to the absorption of iron is probably of potential significance. In the etiology of the hypochromic anemia of hookworm disease, however, the much lower incidence of gastric anacidity correspondingly diminishes the importance of this factor.

Since the patients with anemia unassociated with hookworm infection were observed in a community in which sprue is common, it is possible that the defective dietary background considered to be an etiological factor in sprue (52) (53) (54) has an influence upon the patient with hypochromic anemia. As was stated above, the distinction between cases of sprue with hypochromic anemia and cases of hookworm disease with lingual and intestinal symptoms was necessarily arbitrary in some instances. Indeed, of the 47 patients selected, 8 had a history of or showed signs of glossitis and 24 had had moderately severe diarrhoea on certain occasions preceding the present illness. The fact that transient diarrhoea is a common occurrence in people in the tropics who are apparently in good health otherwise, should not set aside its potential significance as a mechanism leading to the faulty absorption of hematopoietic factors in the light of the observations of Keefer (39) (47) and others. In studying patients with sprue, in certain instances the effectiveness of orally administered liver extract was found to diminish as time went on, although its activity by parenteral injection remained good (55) (56). This was regarded as evidence of a progressively decreased absorption of the orally administered active principle. By analogy it is perhaps reasonable to assume that the intestinal tract of certain individuals who live on defective diets and who harbor hookworms may become less permeable to iron. Such a supposition is clearly necessary in comprehending the etiology of certain cases of hypochromic anemia not associated with hookworm infection. Here again the oral dosage of iron salts necessary to produce a given hematopoietic effect varies from patient to patient, as does the dosage of liver extract in different patients with pernicious anemia or sprue. Rotter (57) described the occurrence of morphological change in the small intestine in hookworm disease. He stated that the submucosa was thickened, with hyaline changes of the connective tissue and deposit of hemosiderin. The mucosa may be from 3 to 5 times as thick as the normal, with areas of atrophied mucosa containing very few glands. At any rate, the association of changes in the gastrointestinal tract with other types of hypochromic anemia is so common that in the face of the available evidence, such changes must surely be considered as a factor in the etiology of the hypochromic anemia associated with hookworm infestation.

IV. OBSERVATIONS CONCERNING THE TREATMENT OF THE ANEMIA

Although a few reports on the effect on administering hematopoietic substances upon the anemia associated with hookworm disease existed in the literature at the time that these studies were undertaken, the attention of those engaged in treating patients with hookworm disease was largely taken up with the elimination of the parasite. A careful analysis of the relative effects of the removal of the parasites, and of therapy with improved diets, with iron and with certain liver extracts has not to our knowledge, however, previously been carried out under controlled conditions in hospitalized patients as well as in field experiments. Since the publication of the preliminary report of this paper (11), Suárez (58) and Cruz (59) have confirmed certain of the results described here in detail.

A. The effect of removing the hookworms. The effectiveness of removing the hookworm burden in bringing about relief of the anemia has not always been uniform in the experience of others (8) (19) (60), although the elimination of the parasites has generally been considered necessary to a cure. Ashford, King, and Igaravídez (8) concluded from their observations in Puerto Rico that the elimination of the hookworms would bring about rapid relief of the anemia in the majority of instances. The present observations present a striking contrast to their conclusions since the effect of removal of the parasites without other therapy was, at best, slight. The essentially negative effect of removing the parasites from 12 patients who were maintained under basal conditions in the hospital has been described in detail above (see table 2 and figure 3). Below are described the confirmatory results of similar observations in the field, which are summarized in table 9 and figures 5 and 6.

B. The effect of iron salts. Although Ashford, King, and Igaravídez (8) did not consider therapy with iron of any particular importance, compared to elimination of the hookworms, in the relief of the anemia, a careful scrutiny of the protocols of their observations suggests that the iron administered in many instances was of importance in bringing about the rapid improvement observed. This interpretation finds support in the observations of others. Boycott (60) observed improvement of the blood picture in hookworm disease with iron and arsenic therapy. Day and Ferguson (19) showed striking improvement on giving iron. Kobayashi (61) and Yang and Keefer (62) demonstrated the positive effect of the administration of iron upon hematopoiesis in a few patients without removing the parasites. As described above, the daily administration of 6 grams of ferric ammonium citrate *without removal of the parasites* was attended in our experience by rapid relief of the anemia. The data for such observations upon 27 patients are presented in tables 3 and 4. In table 4 are also shown the results of similar therapy upon the hemoglobin values of 6 patients whose hookworm burden had previously been removed. The average increases of hemoglobin in the two series during the first thirty days were 25 and 31 per cent respectively. These values compare favorably with the results of iron therapy in other types of hypochromic anemia; and were confirmed by the studies in the field described below. In the group of 6 patients whose hookworm burden was not removed the average gain of hemoglobin in thirty days was 38 per cent, whereas in the group of 5 patients whose hookworms were eliminated the gain was 34 per cent in thirty days.

Thus, the lesson is clear, that the removal of the hookworm burden has in itself no especial effect upon hematopoiesis, whereas the administration of iron salts, irrespective of the presence of the parasite, at once brings about rapid hemoglobin regeneration. Moreover, the increase in hemoglobin values is coincident with remarkable clinical improvement. Marked subjective improvement was sometimes experienced within two or three days. Thereafter the lassitude rapidly diminished, the color and strength improved, the appetite increased, edema disappeared, and the circulatory signs of anemia were progressively relieved. Moreover, the gastrointestinal symptoms of the disease, sometimes ascribed to the irritating effect of the parasites, were progressively relieved as the blood values rose, whether the hookworms were removed or not. Improvement of the glossitis, as in idiopathic hypochromic anemia, was also observed. This is the more remarkable since the only change in therapy had been the addition of iron salts to the basal or native diets. On the contrary, the effect of simply removing the parasites improved the clinical condition of the patients no more than it did their blood values. They remained pale, weak, and apathetic.

It will be noted that in many instances the hemoglobin values in the tables had not reached normal values by the end of the periods of iron administration. This is due in part to the fact that the data do not extend beyond the termination of the particular type of observation under consideration, or that the patients left the clinic or hospital. For obvious reasons the effects of various procedures subsequently carried out on certain patients could not be shown in detail in the tables. However, although the patients regarded themselves as entirely well, the gain in hemoglobin, after a value of about 70 per cent was reached as a result of the administration of iron, was often slow as in other types of hypochromic anemia. The effect of removing the parasites or of giving in addition aqueous extract of liver was, therefore, tried in certain patients whose hemoglobin values seemed to be making slow progress. Neither of these procedures could be demonstrated to have any particular effect, and it became evident that time was necessary in many instances to complete the results of iron therapy. Furthermore, if permanent changes in the absorptive capacity of the gastrointestinal tract have occurred, as apparently exist in other types of hypochromic anemia, it is probably necessary in certain patients to continue a maintenance dose of iron even after the blood values are normal (63).

C. The effect of various extracts of liver. Cohn, Minot, and their collaborators (64) developed the first fractions of liver for the treatment of pernicious anemia. Based on their methods the processes now employed consist essentially in the following steps: Beef or pig liver is finely minced and extracted with water. Upon acidification to pH 5 and heating to 80°C. a copious precipitate results. The remaining liquid is then concentrated in vacuo to a syrupy consistency. This fraction is effective in the treatment of pernicious anemia and was successfully used by Suárez (9) in the treatment of the anemia of hookworm disease. When the syrup is poured into alcohol in such amounts that a 70 per cent concentration of alcohol by volume is obtained, a second precipitate results. This fraction has been found effective by Whipple and his coworkers (65) in the dietary-blood loss anemia of dogs. A considerable portion of the material soluble in 70 per cent alcohol may be precipitated by the addition of absolute alcohol until a concentration of 95 per cent by volume is reached. The dried precipitate, which is the original "fraction G" of Cohn, is highly effective in pernicious anemia but has little or no effect in hypochromic anemia (13) (63).

Accordingly certain commercial preparations of these fractions of liver were administered to suitable patients under the usual controlled conditions in the hospital. The effect of the simple aqueous concentrate[2] previously used by Suárez (9) was evaluated in dosages known to be effective in pernicious anemia. Observations were made on 15 patients. In table 6 are shown in detail the data concerning red blood cells, hemoglobin and reticulocytes during observations on 6 patients, cases 1, 8, 26, 27, 61, and 64. No anthelmintic treatment was given. During periods of several days the amount of Liver Extract E-29 derived from 340 grams of liver was given daily to each patient, except to case 26 (a boy of 9 years) who received the amount derived from only 240 grams. With the exception of case 64, there was in each patient an increase of reticulocytes followed by an increase of hemoglobin. In cases 8, 26 and 64, in immediately subsequent periods following the discontinuance of Liver Extract E-29, the daily administration of ferric ammonium citrate produced second reticulocyte peaks. In table 7 are summarized the data upon hemoglobin production in 15 patients treated daily with the amount of Liver Extract E-29 derived from 340 grams of liver. These data were derived from observations of the 6 patients just cited and 9 others observed as out-patients. The average increase of hemoglobin for the group in thirty days was only 13 per cent compared with 25 per cent in a group of 27 other patients receiving 6 grams of ferric ammonium citrate daily (see table 4). These observations confirm the activity of Liver Extract E-29 in this type of anemia noted by Suárez, but indicate the greater effectiveness of the iron salt, at least in the dosages employed. The relative effectiveness of these substances thus resembles the results in other types of hypochromic anemia.

The fraction of liver which is insoluble in 70 per cent alcohol is relatively ineffective in the treatment of pernicious anemia. It corresponds, however, to the fraction shown by Whipple and his associates (65) to have hemoglobin-producing ability in anemic dogs. This substance is commercially available in dry form as Liver Extract No. 55, except that in this particular preparation approximately 0.5 gram of ferric ammonium citrate has been added to the amount of extract derived from 100 grams of liver. Without the iron, this material[3] was administered daily to 7 patients in doses of 12 grams, representing the extract derived from 400 grams of liver. In 3 patients there was no significant effect upon hematopoiesis during periods of ten days as illustrated by the data from cases 22 and 51 shown in table 6. In the other 4 patients of this series, there was a moderate effect upon hematopoiesis from feeding this liver fraction (see case 36). In all instances there was a second increase of reticulocytes when the patients were given daily 2 grams of ferric ammo-

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[2] This fraction is commercially available as Liver Extract E-29, kindly supplied by Valentine's Meat Juice Company.

TABLE 7

Effect upon hemoglobin production of daily administration of liver extract E-29 derived from 340 grams of liver

Hookworms not removed

CASE NUMBER	HEMOGLOBIN PERCENTAGES AT 10-DAY INTERVALS						
	0 days	10 days	20 days	30 days	40 days	50 days	60 days
1	34	40	46	52			
2	15	18	30				
8	35	46					
15	28	34	38	45	47	51	
19	22	32	40	44			
27	28	41	45				
31	31	41	40	42	42		
44	23	24	28	33			
45	28	35	38	40			
50	38	39	44	50	51		
54	36	40	40	42	46	49	53
61	20	27					
64	42	45					
75	34	39					
Averages....	30	36	39	43	47	50	53

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[3] Kindly supplied without added iron by Eli Lilly and Company.

nium citrate, which is the amount of that iron salt added to each 12 grams of the liver fraction in the preparation of Liver Extract No. 55. Thus, the administration of the fraction of liver precipitated by 70 per cent alcohol had relatively slight hematopoietic effects in contrast to the action of the relatively small dose of ferric ammonium citrate. The effect of 2 grams of ferric ammonium citrate upon red blood cell and hemoglobin values was striking but less than that of 6 grams of the same preparation. (See case 51.)

In considering the activity of these liver extracts, the fact that a certain amount of iron was naturally present is possibly of significance. The daily dose of Liver Extract E-29, derived from 340 grams of liver, probably contained between 8 and 25 mgm. of iron. This would correspond to 50 to 156 mgm. of ferric ammonium citrate. The smaller dose would scarcely be of significance, but the effect of the larger amount might be detectable. In the daily dose of Liver Extract No. 55, derived from 400 grams of liver, about 34 mgm. of iron were present according to one analysis. This would correspond to about 210 mgm. of ferric ammonium citrate, which would probably have a slight effect and might, in some part, account for the activity of the material when administered even without the iron added in its commercial preparation.

The fraction of liver which is soluble in 70 per cent alcohol but insoluble in 95 per cent alcohol by volume, is potent in pernicious anemia in daily doses of the amount derived from 300 grams of liver. Liver Extract No. 343, N. N. R., represents a commercial preparation of this fraction. The data for red blood cell, hemoglobin and reticulocyte values from observations on cases 27 and 61 are shown in the first periods of table 6. During periods of ten days the daily oral administration of the extract[4] derived from 300 grams of liver was without detectable effect in these and in two other patients. In case 27 the reticulocytes were originally elevated, but did not increase during the administration of this liver extract, as occurred immediately thereafter in both cases 27 and 61 with the administration of Liver Extract E-29. The daily intramuscular injection of the amount of Liver Extract No. 343, N. N. R., derived from 10 grams of liver has the

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[4] Kindly supplied by Eli Lilly and Company.

TABLE 8

Comparison of effects upon hematopoiesis of addition of meat and milk and of ferric ammonium citrate to basal diet without removal of hookworms

	CASE 29			CASE 35			CASE 53			CASE 58			CASE 62			CASE 65			CASE 82			CASE 83		
	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes	Red blood cells	Hemoglobin	Reticulocytes
First periods. Daily administration of 300 grams of meat and 1500 cc. of milk																								
days	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*	*millions*	*per cent*	*per cent*
0	2.91	25	2.6	3.28	47	2.6	1.81	31	0.8	1.94	24	6.0	2.14	32	3.0	1.73	19	2.6	2.88	37	3.6	3.71	46	1.8
2	2.81	28	4.8	3.11	43	2.4	2.03	28	2.8	2.30	28	10.8				1.46	19	2.4	2.70	37	3.4	3.60	47	1.8
4	2.92	28	3.8	3.59	47	4.0	2.40	28	2.8	2.33	24	5.4	1.81	26	4.6	1.50	20	4.0	3.04	42	3.6	3.67	50	3.2
6	2.75	28	5.8	3.43	46	2.2	2.08	25	3.0	2.25	25	5.2	1.79	29	4.2				3.00	36	3.2	3.99	53	3.0
8	3.10	32	3.6	3.40	44	2.4	2.02	21	2.0	2.20	21	6.4	1.82	27	5.4	1.71	19	3.0	2.68	36	3.8	3.92	54	2.2
10	3.34	37	3.8	3.39	44	2.4	1.76	20	4.4	2.08	22	3.8	1.59	26	4.8	1.38	18	3.4	3.24	37	2.4	3.98	51	2.8
12	3.33	36	2.8	3.50	48	1.8	1.89	23	4.0	2.11	23	5.0	1.57	25	4.2	1.58	20	2.8	2.89	37	3.2	3.73	45	2.6
14	2.81	30	3.4				1.86	22	4.6	2.34	26	4.2	1.58	24	4.2	1.56	20	4.6	3.11	39	3.6	3.99	53	2.2
16	2.40	28	4.6	3.38	42	1.6	1.93	21	4.6	—	—	—	1.46	23	4.8	—	—	—	2.67	36	2.8	4.03	50	2.6
18	—	—	—	3.39	41	1.8	—	—	—				1.46	22	6.2				2.76	38	2.2	3.76	48	1.8
Second periods. Daily administration of 6 grams of ferric ammonium citrate																								
0	2.40	28	4.6	3.39	41	1.8	1.93	21	4.6	2.34	26	4.2	1.46	22	6.2	1.56	20	4.6	2.76	38	2.2	3.76	48	1.8
2	2.48	25	6.6	3.56	44	4.0	1.93	21	8.6	2.30	26	7.4	1.39	22	3.0	1.98	21	4.6	2.86	38	1.4	3.92	54	1.8
4	2.71	30	6.2	3.91	50	*6.8*	1.85	23	12.0	2.58	29	15.0	1.45	24	3.0	2.12	23	10.2	3.05	41	3.0	4.10	51	1.8
6	2.73	32	*7.4*	3.97	57	3.4	2.21	33	*15.8*	2.67	37	*16.0*	1.87	30	3.8	2.07	28	*12.0*	3.20	43	*4.0*	4.24	57	2.6
8	2.91	37	6.6	4.36	59	3.2	2.54	34	13.8	3.14	40	12.8	2.10	37	8.6	2.09	29	5.6	3.60	50	3.4	4.60	60	1.2
10	2.82	41	4.6	4.59	61	4.8	2.30	38	9.8	3.25	44	6.6	2.05	35	*10.2*	2.25	31	5.2	4.15	49	2.2	4.32	59	1.0

12	2.70	46	3.8	4.99	63	2.6	1.98	44	10.2	3.48	52	8.2				2.88	38	4.4	4.10	51	1.8			
14	3.05	44	3.4	4.70	70	4.0	2.76	43	7.4				1.94	40	6.2									
16	3.14	42	1.2				3.15	47	7.0				2.00	41	6.0				4.20	56	1.8	4.42	62	1.2
18	3.54	49	2.0				2.82	46	5.8							3.04	47	6.0				—	—	—
20	3.69	51	1.2				3.41	52	7.0	3.61	63	3.0				—	—	—	4.00	53				
22	—	—	—				3.82	58	3.0															
24																								
26							3.36	50	1.6				2.88	48	2.0									
28				4.79	70	0.6				4.03	66								4.05	58	2.4			
30				—	—	—													—	—	—			
32																								
34							3.92	59		3.93	67	0.6	3.11	51	2.8									
Ova per cgm. stool....	158			237			931 (2,231 worms)*			113 (155 worms)*			607 (1,424 worms)*			601			124			Positive		

* Number of worms subsequently removed.

hematopoietic effect in pernicious anemia of the daily oral administration of the same extract derived from at least 300 to 400 grams of liver. This extract[6] injected daily in amounts derived from 10 grams of liver had no effect on hematopoiesis in 8 patients, as illustrated by the data from the first periods of cases 5, 9 and 11, shown in table 3. The negative effect of this material in the hypochromic anemia of hookworm disease thus corresponds with the results in other types of hypochromic anemia.

D. The effect of improved diet. The deficient quality of the diet of the majority of these patients has been discussed above. It therefore seemed logical to evaluate its etiological and therapeutic implications by observing the effect upon the anemia of improving the diet. The basal diet in the hospital of 8 patients was accordingly improved by the daily addition of 1500 cc. of milk and 300 grams of lean beef. It was felt that these two substances would yield an excellent supply of animal protein, would provide certain vitamins, and serve as sources of iron and calcium. In table 8 are shown the detailed results of these dietary additions upon hematopoiesis. No treatment for the elimination of the parasites was given until after the completion of the observations. It will be seen that within periods of time varying from fourteen to eighteen days, no significant effect upon reticulocyte production, hemoglobin or red blood cell values was produced. Case 58 was apparently running an elevated reticulocyte count throughout the period of observation. Although in a few instances there were gradual increases of reticulocytes of slight degree, the absence of any significant effect produced by the improved diets is clearly brought out, upon the return to the basal diet, by the positive effect of the administration of ferric ammonium citrate in daily doses of 6 grams. In every instance, rises of red blood cell and hemoglobin values resulted, associated with increases of reticulocytes. The negative effect of the administration of the improved diet, contrasted with the positive effect of iron upon reticulocyte and hemoglobin production in cases 53 and 62, is graphically presented in figure 4.

V. OBSERVATIONS IN THE FIELD

The results in hospitalized patients of these observations upon the relative therapeutic efficacy of removal of the hookworms, of improved diets, and of iron therapy were confirmed in general by the field observations in Cidra on a group of 32 patients with initial hemoglobin values of less than 50 per cent of normal, and hookworm ova in the stools. These observations are being reported elsewhere in detail (12). The patients were largely employees of a local company, and lived in small houses scattered over an area of several miles. Men, women and adolescent children were selected at random, if found to be sufficiently anemic. Constant supervision was exercised to see that the medicine and food dispensed were actually taken by the proper individuals in the prescribed amounts.

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[6] Kindly supplied by Eli Lilly and Company.

The 32 patients were divided arbitrarily into two main groups, A and B, of 16 patients each. Those in group A were treated three times with carbon tetrachloride and oil of chenopodium followed by a saline purge before the beginning of the experimental period. By examination of the stools the elimination of the parasites was assured and maintained by re-treatment at approximately monthly intervals. In a few of these patients, despite three or more treatments, a few ova were constantly found in the stools. The patients of group B received no eliminative treatment, and the presence of a persistent hookworm infection was confirmed by examination of the stools for ova at frequent intervals. Each of the two main groups, A and B, was divided into three subgroups, I, II and III respectively. The patients in groups A-I and B-I had no change made in their diets during the whole course of the observations. The patients in groups A-II and B-II with the beginning of the experimental period were given daily 300 grams of lean meat and 1500 cc. of milk in addition to their usual diet. They were also encouraged to eat more fruit and leafy vegetables. The patients in groups A-III and B-III received no change in diet, but were given daily by mouth 6 grams of ferric ammonium citrate in the form of a 50 per cent aqueous solution. The blood studies consisted of capillary red blood cell and hemoglobin determinations, made at intervals of approximately a week during both the control and the experimental periods. The hemoglobin readings were invariably performed with the same Sahli instrument by the same observer.

For convenience in presenting the results of these observations, the hemoglobin determinations made upon each patient were plotted

TABLE 9

Comparison of effects upon hemoglobin production of removal of hookworms, of improved diet, and of administration of ferric ammonium citrate, in field observations at Cidra

Figures in table are hemoglobin percentages

GROUP NUMBER	CASE NUMBER	DAYS BEFORE			THERAPY	DAYS AFTER BEGINNING OF EXPERIMENTAL PERIODS									
		30	20	10		0	10	20	30	40	50	60	70	80	90
Group A. Hookworms removed															
A I	1			47	Diet not changed	50	52	46	49	57	56	52	54	57	56
	2		21	27		30	33	37	38	39	42	45	48	47	58
	3		34	30		28	31	34	36	32	30	28	45	41	
	4		39	40		40	41	40	42	41	42	48	52	53	48
	5			40		42	41	40	41	42	45	42			
	6		31	33		36	34	41	33	32					
	Averages		31	36		38	39	40	40	41	43	43	50	50	54
A II	7			30	300 grams meat, 1500 cc. milk added daily	31	35	37	41	43	41	44	49	51	48
	8		30	33		34	34	32	31	35	36	42	50	46	46
	9	34	34	35		31	31	29	28	28	40	38	36	29	32
	10	28	29	28		28	28	27	26	25	23	21	19	21	22
	11	12	9	14		18	17	19	21	25	26	29	24	28	30
	Averages	25	28	28		28	29	28	29	31	33	35	36	35	36
A III	12			26	Diet not changed; 6 grams ferric ammonium citrate daily	25	37	58	72	77	75	75			
	13			33		36	46	54	78						
	14	36	36	37		37	62	65	66	69	87	80	80		
	15	21	24	26		28	36	40	44	53	72	72	81		
	16			22		29	42	51	70	70	71	71	72		
	Averages	29	30	29		31	45	54	66	67	76	75	78		
Group B. Hookworms not removed															
B I	17					38	36	24	22	25	25	25			
	18				Diet not changed	28	20	19	19	22	20	17	24		
	19					30	31	28	24	37	32	33	31	27	
	Averages					32	29	24	22	28	26	25	28	27	
B II	20		39	35	300 grams meat, 1500 cc. milk added daily	36	40	38	40	46	46	46	46	48	48
	21	43	43	43		43	39	49	48	45	46	44	48	42	48
	22	44	43	43		43	46	40	32	36	36	31	32	34	31
	23	38	34	31		28	30	35	33	30	28	27	26	29	26
	24			29		23	25	18	13	11	8				
	25		31	30		31	35	35	38	33	33	26	25	24	20
	Averages	42	40	36		34	36	36	34	34	33	35	35	37	35

TABLE 9—*Concluded*

GROUP NUMBER	CASE NUMBER	DAYS BEFORE			THERAPY	DAYS AFTER BEGINNING OF EXPERIMENTAL PERIODS									
		30	20	10		0	10	20	30	40	50	60	70	80	90
Group B. Hookworms not removed—*Concluded*															
B III	26	19	18	18	Diet not changed; 6 grams ferric ammonium citrate daily	20	52	59	80	66	68	76	81		
	27		29	30		29	51	61	73	64	69	77	78		
	28		25	26		29	40	58	66	72	72	69			
	29					39	44	59	68	71	73	78	82		
	30	28	29	30		32	43	60	64	66	68	83	83	84	87
	31	43	38	35		36	57	62	70	73	74				
	32	34	36	37		38	57	70	68	67	73	75			
	Averages	31	29	29		32	49	61	70	68	71	76	81	84	87

against days, and from the curve so constructed the values at ten-day intervals were defined and are presented in table 9. From these data the average hemoglobin values for each group were calculated for ten-day intervals, and the results are plotted in figure 5. The average hemoglobin percentage of the 6 patients of group A-I, who had the hookworms removed but received no other therapy, shows a gradual upward trend, but in one hundred ten days the hemoglobin gain was only about 20 per cent. The average hemoglobin percentage of the 3 patients of group B-I, who received no treatment of any kind, decreased 5 per cent in a period of eighty days. The average hemoglobin percentage of the 5 patients of group A-II, who received the improved diet after having the parasites eliminated, showed an increase of 8 per cent in ninety days. The average hemoglobin percentage of the 6 patients of group B-II, who received the improved diet without removal of the parasites, was a few percent less at the end of one hundred twenty days. These results are then consistent with the assumption that by the removal of the parasites a constant source of blood loss was abolished, which resulted in the ability of the patients to gain hemoglobin very slowly, in contrast to the slow descent of the hemoglobin values of the patients still harboring parasites. They also confirm entirely the facts observed under controlled conditions in the hospital, that the removal of the parasites alone results in little improvement. The improved diet apparently was unable significantly to influence the rate of hemoglobin formation as already demonstrated by the observations on other patients in the hospital.

The data for the red blood cell values of individual patients were treated in a fashion similar to that applied to the data on hemoglobin and are shown in table 10. The removal of the parasites without other therapy permitted within thirty days moderate increases of erythrocytes in contrast to the insignificant effect upon hemoglobin formation. During periods as long as ninety days, somewhat greater increases took place. During one hundred ten days the average increases of red blood cells in groups A-I and A-II were respectively 1.6 and 2.0 million per cubic millimeter. In the corresponding groups in which the parasites were not removed, there were respectively decreases of 0.7 million in eighty days in group B-I and 0.4 million per cubic millimeter in one hundred ten days in group B-II. The averages of the combined red blood cell values of the 11 patients of groups A-I and A-II, and of the 9 patients of groups B-I and B-II, are plotted in figure 6. The average hemoglobin values of these two groups of patients are also plotted in the same figure. These data are thus derived from all the patients not receiving iron; and the differences between the two groups represent the effectiveness of removing the hookworm burden. In one hundred ten days the average increases of red blood cells and of hemoglobin of the 11 patients whose hookworm burden had been removed were respectively 1.8 million per cubic millimeter and 20 per cent. In one hundred days there were decreases of the average red blood cell and hemoglobin values of the 9 patients whose hookworm burden had not been eliminated, of 0.55 million per cubic millimeter and of 7 per cent, respectively. It is thus clear that the effect of removing the parasites is greater upon red blood cell than upon hemoglobin production, with a resultant lowering of the color index. This implies that the available hemoglobin has been redistributed among a larger number of corpuscles.

However, it was only when iron was given that both hemoglobin and red blood cell values were rapidly augmented, as shown by the data from the patients of groups A-III and B-III, given in tables 9 and 10. Under these circumstances, the presence of the parasites apparently had no detectable effect upon the production of either red blood cells or hemoglobin. Only by the use of iron was the oxygen-

TABLE 10

Comparison of effects upon red blood cell production of removal of hookworms, of improved diet, and of administration of ferric ammonium citrate, in field observations at Cidra

Figures in table are red blood cells in millions per cubic millimeter

GROUP NUMBER	CASE NUMBER	DAYS BEFORE			THERAPY	DAYS AFTER BEGINNING OF EXPERIMENTAL PERIODS									
		30	20	10		0	10	20	30	40	50	60	70	80	90
					Group A. Hookworms removed										
A I	1				Diet not changed	3.8	4.1	4.4	4.5	4.5	4.5	4.3	4.1	4.0	4.3
	2		1.9	2.4		2.9	3.4	3.7	3.5	3.5	3.5	4.1	4.8	4.4	4.3
	3		1.8	1.9		2.1	2.7	2.5	3.3	3.6	3.5	3.4	3.5	3.2	2.9
	4	2.2	2.7	3.2		3.7	3.8	3.6	3.8	4.1	4.4	4.1	3.9	3.7	3.5
	5			3.2		2.9	3.1	3.3	3.4	3.6	4.2	3.2			
	6		2.6	2.8		3.0	3.5	3.2	3.1	3.8					
	Averages	2.2	2.3	2.7		3.1	3.4	3.5	3.6	3.8	4.2	3.8	4.1	3.8	3.8
A II	7			3.4	300 grams meat, 1500 cc. milk added daily	3.8	3.9	3.5	3.8	3.6	3.2	3.8	4.1	4.0	4.6
	8		3.0	3.1		3.4	3.6	3.8	3.7	3.8	4.2	3.7	4.3	4.3	4.8
	9	3.0	3.5	3.9		3.0	2.6	3.8	3.4	3.3	3.7	3.5	3.4	2.5	3.2
	10	2.3	2.3	2.3		2.4	3.4	3.1	2.9	2.7	2.4	2.2	2.1	2.2	
	11	1.3	1.3	1.3		1.6	1.7	1.8	1.9	2.1	2.2	2.9	2.6	2.7	
	Averages	2.2	2.5	2.8		2.8	3.0	3.2	3.1	3.1	3.1	3.2	3.3	3.1	4.2
A III	12		1.7	1.7	Diet not changed; 6 grams ferric ammonium citrate daily	2.2	2.6	2.9	3.3	4.0	4.1	4.2	4.0		
	13			3.5		3.3	3.8	4.0	3.3						
	14	3.5	3.6	3.6		3.8	4.3	4.5	4.6	4.7	4.6	4.5	4.2	4.0	
	15	2.6	2.7	2.9		3.1	3.0	3.4	3.6	3.8	4.2	3.5	4.3		
	16			2.7		3.0	3.8	3.8	3.9	4.6	4.3	3.8	4.4		
	Averages	3.1	2.7	2.9		3.1	3.5	3.7	3.7	4.3	4.3	4.0	4.2	4.0	
					Group B. Hookworms not removed										
B I	17				Diet not changed	3.0	2.6	2.5	2.9	3.5	3.3	3.2			
	18					2.4	1.5	1.4	1.6	1.6	1.3	1.1	2.1		
	19					3.5	3.0	3.2	3.2	2.8	2.7	2.0	2.0	2.3	
	Averages					3.0	2.4	2.4	2.6	2.6	2.4	2.1	2.1	2.3	
B II	20	4.0	4.0	3.9	300 grams meat, 1500 cc. milk added daily	3.9	3.9	3.8	3.8	3.8	3.9	4.2	4.1	4.2	3.9
	21						3.3	2.8	2.8	3.2	3.6	3.2	3.7	3.5	3.5
	22	3.2	3.4	3.6		3.8	3.9	3.5	3.2	3.4	3.3	2.8	3.5	2.7	2.7
	23	2.3	2.3	2.2		2.2	2.4	3.0	3.1	2.8	2.3	2.1	2.2	2.1	1.4
	24					1.9	1.6	1.4	1.3	1.2	1.0				
	25		3.2	3.5		3.9	3.9	3.8	3.6	3.3	3.6	3.2	2.6	2.4	2.7
	Averages	3.2	3.2	3.3		3.1	3.2	3.1	3.0	3.0	3.0	3.1	3.2	3.0	2.8

TABLE 10—*Concluded*

GROUP NUMBER	CASE NUMBER	DAYS BEFORE			THERAPY	DAYS AFTER BEGINNING OF EXPERIMENTAL PERIODS									
		30	20	10		0	10	20	30	40	50	60	70	80	90
					Group B. Hookworms not removed—*Concluded*										
B III	26			1.6	Diet not changed; 6 grams ferric ammonium citrate daily	1.9	2.3	3.0	3.7	4.3	4.6	4.8	4.9	4.9	
	27	2.8	2.8	2.7		2.6	2.7	3.7	3.9	3.7	3.4	3.7	5.2	4.7	
	28					3.1	3.5	4.5	4.8	4.6	3.7	4.5	4.2		
	29			2.8		3.2	3.6	3.9	4.0	4.1	4.8	4.8	4.8		
	30					2.4	3.6	3.9	4.0	4.1	4.5	5.0	4.7	4.4	3.7
	31					3.0	3.3	4.3	5.4	5.8	5.9	5.1			
	32					3.6	3.9	4.2	3.9	3.8	3.9	4.3			
	Averages	2.8	2.8	2.4		2.8	3.3	3.9	4.2	4.4	4.4	4.7	4.8	4.7	3.7

carrying capacity of the circulation significantly and rapidly improved, and the symptoms of the anemia consequently alleviated. The average hemoglobin values for the 5 patients of group A-III, who were treated for the parasites and then given daily 6 grams of ferric ammonium citrate, as well as the average hemoglobin values for the 7 patients of group B-III, who received the same amount of iron salt but no treatment for the removal of the hookworms, increased rapidly after the beginning of the administration of the iron ammonium citrate. The average gains of hemoglobin in each group are comparable, and amount to 34 and 38 per cent respectively during the first thirty days of iron therapy. During the first sixty days of iron therapy each group gained 44 per cent of hemoglobin. It is thus clear from these observations that the presence of the parasites had no detectable inhibiting effect upon the production of hemoglobin in response to iron therapy. This result confirms the observations conducted under controlled conditions in the hospital, and demonstrates the practicability of therapy with iron in the field.

VI. DISCUSSION

The observations reported above, concerning the etiology of the anemia of hookworm infestation, at least in Puerto Rico, make it clear that as far as the morphology of the blood picture and of the bone marrow is evidence of similarity, the anemia resembles various types of hypochromic anemia unassociated with hookworm infection. Patients showing no evidence of hookworm infection but with identical blood pictures and physiological responses to hematopoietic substances were encountered in Puerto Rico. Today the hypochromic anemias are considered to be mainly due to defective blood formation, particularly to defective hemoglobin production. Apparently the immediate cause is a lack in the body, mainly of available iron (21). The remarkable effectiveness of large doses of iron ammonium citrate in the relief of the hypochromic anemia of hookworm disease does not differ from that observed in the therapy of other types of hypochromic anemia in which faulty diets, gastrointestinal changes, and blood loss clearly play rôles of varying importance in bringing about an iron deficiency. These possible etiological factors were accordingly evaluated as far as was possible. Strikingly defective diets, some glossitis, a moderate percentage of gastric anacidity, and a good deal of diarrhoea were found. The reduction of available hematopoietic material in the body through blood loss rests upon a firm basis from experiments on animals, and was confirmed by our observations on the hematopoietic effect of injected red blood cells. Since it was impossible to demonstrate any direct inhibitory effect of the parasite upon the function of the bone marrow, it is our belief that the influence of the parasite in the production of anemia depends solely upon its ability to cause chronic blood loss. Blood loss due to *Necator americanus* undoubtedly occurs, although it is probably not so great as that due to *Ancylostoma duodenale*. The difficulty of reconciling the varying degrees of anemia or even apparently perfect health, which may exist in association with a given amount of hookworm infection, is, however, easily surmounted if it is considered that the other factors, which we have examined, play contributory rôles in reducing the available supply of hematopoietic substances in the body. Despite the fact that beans and peas are apparently a good source of iron, the obvious limitation of the diet in animal protein and in vitamins A, B, and D, brings again into prominence the etiological relationship of dietary defects, which were considered important before the discovery of the parasite. The obviously disturbed condition of the gastrointestinal tract of so many of these patients is sufficient to suggest difficulty with the assimilation of hematopoietic substances. Such a disturbance can be shown to exist in other hypochromic anemias, especially when gastric achlorhydria is present. Not all the patients with hypochromic anemia associated with hookworm infection stand entirely apart from patients with sprue, which is also common in Puerto Rico. Our selection was arbitrarily made on the basis of the hypochromic type of blood picture and the finding of ova in the stools, but patients with sprue are encountered with hypochromic blood pictures. The symptoms of sprue strongly suggest an involvement of the intestinal tract in the etiology of the process. The gastrointestinal disturbances and the macrocytic anemia are probably due to a deficiency in the diet of a heat-stable substance associated with many sources of the vitamin B complex (52) (53) (54). Changes in the gastric function and in the intestinal permeability (55) (56) have been demonstrated in sprue; and it is possible that in the dietary background of the sufferers from hypochromic anemia there are defects which have led to changes in the absorptive function of the alimentary canal with respect to iron. In this way certain individuals could become "conditioned" for difficulty in the assimilation of iron, as is clearly the case in other types of hypochromic anemia. The fact that rapid blood production and striking clinical improvement invariably resulted from the addition of iron salts to the basal diet of the patients, indicates that the chief immediate defect leading to the production of the hypochromic anemia is iron. This does not mean that although no significant effect resulted from improvement of the diet alone, dietary defects other than iron may not play a part leading up to the iron deficiency; nor that an increase of the food iron would not be important in preventing the development of the anemia. The presence of other deficiencies is suggested by the fact that a concentrated aqueous extract of liver containing negligible amounts of iron was found to be effective. The possibility of reversing gastrointestinal changes produced by defective diets over long periods of time is obviously more remote than simply overcoming their immediate effects by the administration of large doses of hematopoietic substances. It also is not intended to imply that because the anemia can be totally relieved by the exhibition of iron without removing the parasites, the latter play no part in its production. In varying degrees in different patients the factors of food deficiency, gastrointestinal changes, and blood loss, apparently share the credit for causing this type of hypochromic anemia as in other varieties.

Certain prevailing impressions concerning treatment are contradicted by an analysis of the problem carried out by testing the effect of only *one procedure at a time.* The observation was repeatedly made that the removal of the parasites resulted in little improvement of the blood picture or the clinical condition of the patients sometimes over periods as long as ninety days. In the severely anemic patients who were selected, the supply of hematopoietic substances had already been so greatly reduced that the mere prevention of further blood loss through removing the parasites could perhaps not be expected to bring about remarkable benefit. It is, therefore, possible that our observations do not represent entirely the effectiveness of removing the parasites from less anemic patients. On the other hand, without removal of the parasites, prompt improvement of the blood values and of the clinical condition invariably resulted from the administration of iron salts without other changes in the diet. Thus, it is clear that by direct treatment of the anemia, on which seem to depend the major symptoms of the disease, clinical improvement can be greatly expedited. In the severely anemic patient the administration of anthelmintics may not be unattended with danger, and certainly causes more discomfort than in a patient already restored to health. The importance of measures directed against the parasite is not to be deprecated, but the possibility of a quick return to better health and economic usefulness should aid in securing coöperation in such prophylactic measures as the wearing of shoes, the erection of adequate latrines and the use of better food.

In addition to the cure of the anemia this approach should be useful as a factor in prevention. Since without anemia the presence of the worms is apparently not in itself detrimental to health, it should be possible to employ iron salts as a prophylactic, either alone or preferably in combination with anthelmintics. In patients with little or no anemia the use of improved diets might be more effective than in the already depleted patients who were studied here. It is important to realize, however, that the nutritional state of the patients maintained on a basal diet seemed to be rendered eminently satisfactory by the addition of ferric ammonium citrate alone. For this reason the cost of the treatment necessary to relieve or presumably to prevent the anemia is astonishingly low. A 50 per cent aqueous solution of ferric ammonium citrate (brown scales) was found to keep well in the tropics and could be prepared with very little labor from crystals costing only $2.00 a kilogram. The daily dosage of ferric ammonium citrate for adults should be at least 4 grams, preferably 6 grams for maximum effects. The full amounts should not be given at first, and then only in divided doses after meals. In children smaller doses are desirable. Undoubtedly the prophylactic dose of iron need not be so large or could be administered at greater intervals of time. Observations on this point should be of great practical value. Other preparations of iron are also undoubtedly satisfactory for routine use in correspondingly large dosage. As with other types of hypochromic anemia associated with changes in the gastrointestinal tract, it is probably essential in many instances to continue the administration of iron after the patient has been returned to health, if assimilation of the food iron is not adequate (62). The effect of the liver fractions tested was found to be either negative or inferior to that of iron salts, and their cost far greater. Liver extracts, therefore, appear to offer no advantages over iron in the treatment of this type of hypochromic anemia. It is to be hoped that since the symptomatology of hookworm infection is predominantly that of anemia, the primary importance and simplicity of dealing directly with the anemia will be appreciated by those engaged in the problem.

VII. CONCLUSIONS

1. Based upon a study of 83 patients, the morphological and physiological characteristics of the severe hypochromic anemia associated with hookworm infection in Puerto Rico were found to resemble closely those of other types of hypochromic anemia not associated with hookworm infection.
2. The anemia was considered to be due mainly to insufficient blood production as a result of a deficiency of available iron and other hematopoietic substances in the body.
3. This deficiency is postulated to be produced by *multiple factors*: by defective diets, or indirectly by gastrointestinal changes, or by

blood loss due to the hookworms. Various combinations of these factors are probably involved in different patients.

4. No effect of the presence of the hookworm could be demonstrated other than could be accounted for by its ability to remove blood from the patient.
5. The removal of the hookworms was shown to produce only slight clinical improvement and to have little effect upon hemoglobin production, but considerable effect upon red blood cell production within several weeks.
6. The improvement of the diet, by the daily addition of 1500 cc. of milk and 300 grams of lean beef, was found to have no significant effect upon the anemia.
7. The daily administration of 6 grams of ferric ammonium citrate, *with or without removal of the parasites*, was attended by striking improvement of the blood values and the clinical condition of the patients.
8. The daily administration of a concentrated aqueous extract of liver was shown to have a moderate effect; of a sub-fraction precipitated by 70 per cent alcohol to have a slight effect; and of a sub-fraction soluble in 70 per cent alcohol given either intramuscularly or orally, to have no effect upon blood formation.
9. The practical importance of directing therapy first against the anemia in hookworm disease and secondly against the parasite is pointed out.
10. The medical and economic advantages of using large doses of iron in the prevention and treatment of the anemia of hookworm disease are emphasized.

It is impossible to express our appreciation to all those in Puerto Rico whose knowledge and coöperation aided this work. We are, however, especially indebted to Dr. William R. Galbraith and to Dr. George W. Bachman, through whose kindness the facilities of the Presbyterian Hospital and the Hospital of the School of Tropical Medicine of Columbia University in Puerto Rico were placed at our disposal. We wish to express our gratitude also to Dr. Bailey K. Ashford, to Dr. Ramon M. Suárez and to Dr. Juan A. Pons for their counsel and help in carrying on these observations. It is a particular pleasure to acknowledge the assistance of Mr. Emil Bohnel, Mr. Louis Zetzel and Mr. Amerigo Pomales, as well as the help of many technicians and nurses in carrying out the laboratory and clinical aspects of this work.

VIII. BIBLIOGRAPHY

(1) STILES, C. W.: Report upon the Prevalence and Geographic Distribution of Hookworm Disease (Uncinariasis or Anchylostomiasis) in the United States, Govt. Print. Off., Washington, 1903, U. S. Pub. Health Serv., Hyg. Lab. Bull. No. 10.

(2) HARRIS, H. F.: Ankylostomiasis, the Most Common of the Serious Diseases of the Southern Part of the United States, *Am. Med.*, 1902, **4**, 776.

(3) SMITH, A. J.: Uncinariasis in Texas, *Am. J. Med. Sc.*, 1903, **126**, 768.

(4) CLAYTOR, T. A.: Uncinariosis (Ankylostomiasis). A Further Report of a Case, with Notes upon the Autopsy, *Am. J. Med. Sc.*, 1902, **123**, 28.

(5) SMILLIE, W. G.: Studies on Hookworm Infection in Brazil 1918–1920, *Rockefeller Institute Monograph No. 17*, 1922, 46.

(6) SCHAPIRO, L.: Hookworm Infestation in an Indian (Guaimi) and Non-Indian Population of Panama, *Am. J. Trop. Med.*, 1930, **10**, 365.

(7) ASHFORD, B. K., AND GUTIÉRREZ IGARAVÍDEZ, P.: Uncinariasis (Hookworm Disease) in Porto Rico: A Medical and Economic Problem, Govt. Print. Off., Washington, 1911, Document No. 808.

(8) ASHFORD, B. K., KING, W. W., AND GUTIÉRREZ IGARAVÍDEZ, P.: Report of the Study and Treatment of "Anemia" in Porto Rico, Bur. Print. and Supplies, San Juan, 1904.

(9) SUÁREZ, R. M., MORALES OTERO, P., AND RODRÍGUEZ MOLINA, R.: Estudio de dos casos de anemia, *Bol. Asoc. méd. de Puerto Rico*, 1929, **22**, 5.

(10) SUÁREZ, R. M.: El tratamiento del esprú y el use del extracto acuoso de hígado en su anemia, *Bol. Asoc. méd. de Puerto Rico*, 1931, **23**, 74.

(11) RHOADS, C. P., AND CASTLE, W. B.: Observations on the Etiology and Treatment of the Anemia of Hookworm Disease in Porto Rico, *J. Clin. Inv.*, 1932, **11**, 809.

(12) NUÑEZ, C.: A Study of the Relative Effect of Removal of Hookworms, Dietary Therapy, and Iron in Medicinal Form upon the Anemia of Hookworm Infestation. In press.

(13) WINTROBE, M. M.: The Volume and Hemoglobin Content of the Red Blood Corpuscle, *Am. J. Med. Sc.*, 1929, **177**, 513.

(14) MURPHY, W. P.: An Easy Method of Estimating the Amount of Jaundice by Means of the Blood Serum, *Boston Med. and Surg. J.*, 1926, **194**, 297.

(15) MINOT, G. R., MURPHY, W. P., AND STETSON, R. P.: The Response of the Reticulocytes to Liver Therapy: Particularly in Pernicious Anemia, *Am. J. Med. Sc.*, 1928, **175**, 581.

(16) MINOT, G. R., AND HEATH, C. W.: The Response of the Reticulocytes to Iron, *Am. J. Med. Sc.*, 1932, **183**, 170.

(17) PINEY, A.: The Anatomy of the Bone Marrow: With Special Reference to the Distribution of the Red Marrow, *Brit. Med. J.*, 1922, **2**, 792.

(18) PEABODY, F. W.: The Pathology of the Bone Marrow in Pernicious Anemia, *Am. J. Path.*, 1927, **3**, 179.

(19) DAY, H. B., AND FERGUSON, A. R.: The Treatment of Ankylostoma Anemia, *Lancet*, 1914, **2**, 82.

(20) DAMESHEK, W.: Primary Hypochromic Anemia (Erythro-Normoblastic Anemia), *Am. J. Med. Sc.*, 1931, **182**, 520.

(21) FÜLLEBORN, F., DIOS, R. L., AND ZUCCARINI, J. A.: Bericht über eine im Auftrage der argentinischen Regierung unternommene Reise nach der Provinz Corrientes und nach Paraguay zum Studium der Hakenwurmbekämpfung, *Arch. f. Schiffs- u. Tropen-Hyg.*, 1928, **32**, 441.

(22) ROSENBLUM, M. B., AND MALKOWA, M. N.: Zur Charakteristik der Anämie bei Anchylostoma duodenale, *Folia Haematol.*, 1928, **37**, 197.

(23) SMILLIE, W. G., AND AUGUSTINE, D. L.: Hookworm Infestation. Effect of Varying Intensities on the Physical Condition of School Children, *Am. J. Dis. Child.*, 1926, **31**, 151.

(24) LAMSON, P. D., BROWN, H. W., ROBBINS, B. H., AND WARD, C. B.: Field Treatments of Ascariasis, Ancylostomiasis and Trichuriasis with Hexylresorcinol, *Am. J. Hyg.*, 1931, **13**, 803.

(25) WHIPPLE, G. H.: The Presence of a Weak Hemolysin in the Hookworm and Its Relation to the Anemia of Uncinariasis, *J. Exp. Med.*, 1909, **11**, 331.

(26) DE LANGEN, C. D.: Contributions to the Toxic Origin of the Anaemia and Other Symptoms with Ankylostomiasis, A Study of Lipoids and Anaemia, etc., *Tr. 5th Biennial Congress, for East. Assn. Trop. Med.*, London, John Bale, Sons and Danielsson, Ltd., 1924, 254.

(27) FÜLLEBORN, F.: Epidemiological Observations on Hookworm Infection, *Brit. Med. J.*, 1929, **1**, 755.

(28) WELLS, H. S.: Observations on the Blood Sucking Activities of the Hookworm, Ancylostoma Caninum, *J. Parasitol.*, 1931, **17**, 167.

(29) SMILLIE, W. G.: Hookworm Disease, *Nelson Loose-Leaf Medicine*, Thomas Nelson & Sons, New York, 1920, **2**, 477.

(30) BASS, C. C.: Hookworm Disease, *Practice of Medicine*, Tice, W. F. Prior Co., Hagerstown, Md., 1929, **5**, 79.

(31) DRINKER, C. K.: Diseases of the Blood. The Pathological Physiology of Blood Cell Formation and Blood Cell Destruction, *The Oxford Medicine*, Oxford University Press, New York, 1920, **2**, 509.

(32) MCMASTER, P. D., AND HAESSLER, H.: The Factor Determining the Spread of the Red Marrow during Anemia, *J. Exp. Med.*, 1921, **34**, 579.

(33) MILLER, D. K., AND RHOADS, C. P.: The Effect of Hemoglobin Injections on Erythropoiesis and Erythrocyte Size in Rabbits Rendered Anemic by Bleeding. *J. Exp. Med.*, 1934, **59**, 333.

(34) WHIPPLE, G. H., AND ROBSCHEIT-ROBBINS, F. S.: Assimilation of Blood and Muscle Hemoglobin, *Am. J. Physiol.*, 1927, **83**, 60.

(35) MEULENGRACHT, E.: Large Doses of Iron in the Different Kinds of Anaemia in a Medical Department, *Acta med. Scandinav.*, 1923, **58**, 594.

(36) WITTS, L. J.: The Therapeutic Uses of Iron, *Proc. Roy. Soc. Med.*, 1931, **24**, 7.

(37) HEATH, C. W., STRAUSS, M. B., AND CASTLE, W. B.: Quantitative Aspects of Iron Deficiency in Hypochromic Anemia (The Parenteral Administration of Iron), *J. Clin. Inv.*, 1932, **11**, 1293.

(38) METTIER, S. R., AND MINOT, G. R.: The Effect of Iron on Blood Formation as Influenced by Changing the Acidity of the Gastroduodenal Contents in Certain Cases of Anemia, *Am. J. Med. Sc.*, 1931, **181**, 25.

(39) Keefer, C. S., Huang, K. K., and Yang, C. S.: The Importance of Undernutrition in the Production of Anemia Associated with Chronic Dysentery and Tuberculosis of the Intestine, *Nat. Med. J. China,* 1929, **15**, 743.

(40) Suárez, R. M.: Puerto Rico and Some of Its Health Problems, *Bol. Asoc. méd. de Puerto Rico,* 1930, **22**, 197.

(41) Sherman, H. C.: Chemistry of Food and Nutrition, The Macmillan Co., New York, 1926, Appendix B.

(42) Robscheit-Robbins, F. S.: The Regeneration of Hemoglobin and Erythrocytes, *Physiol. Rev.,* 1929, **9**, 666.

(43) Cook, D. H.: Some Aspects of the Food Problem in Porto Rico, *Porto Rico Rev. Pub. Health and Trop. Med.,* 1927, **3**, 59.

(44) Cook, D. H., and Rivera, B. A.: Significance of Mineral Metabolism: Preliminary Report on the Calcium and Phosphorus Content of Some Porto Rican Food Materials, *Porto Rico J. Pub. Health and Trop. Med.,* 1928, **4**, 65.

(45) McCarrison, R.: Studies in Deficiency Disease, *Oxford Medical Publications,* Henry Frowde and Hodder and Stoughton, London, 1921.

(46) Castle, W. B., Townsend, W. C., and Heath, C. W.: Observations on the Etiologic Relationship of Achylia Gastrica to Pernicious Anemia. III, *Am. J. Med. Sc.,* 1930, **180**, 305.

(47) Keefer, C. S., Yang, C. S., and Huang, K. K.: Anemia Associated with Chronic Dysentery, *Arch. Int. Med.,* 1931, **47**, 436.

(48) Witts, L. J.: The Syndrome of Glossitis, Dysphagia and Anemia, *Guy's Hosp. Rep.,* 1931, **11**, 193.

(49) Castle, W. B., Heath, C. W., Strauss, M. B., and Townsend, W. C.: The Relationship of Disorders of the Digestive Tract to Anemia, *J. Am. Med. Assn.,* 1931, **97**, 904.

(50) Hutter, A. M., Middleton, W. S., and Steenbock, H.: Vitamin B deficiency and the Atropic Tongue, *J. Am. Med. Assn.,* 1933, **101**, 1305.

(51) Davies, D. T.: Studies on Achlorhydria and Anaemia, *Quart. J. Med.,* 1931, **24**, 447.

(52) Castle, W. B., and Rhoads, C. P.: Observations on the Etiology and Treatment of Sprue in Puerto Rico, *Tr. Assn. Am. Physn.,* 1932, **47**, 245.

(53) Strauss, M. B., and Castle, W. B.: The Nature of the Extrinsic Factor of the Deficiency State in Pernicious Anemia and in Related Macrocytic Anemias, *New Eng. J. Med.,* 1932, **207**, 55.

(54) Rhoads, C. P., and Miller, D. K.: The Production in Dogs of Chronic Black Tongue with Anemia, *J. Exp. Med.,* 1933, **58**, 585.

(55) Castle, W. B., Heath, C. W., and Strauss, M. B.: Observations on the Etiologic Relationship of Achylia Gastrica to Pernicious Anemia. IV, *Am. J. Med. Sc.,* 1931, **182**, 741.

(56) Castle, W. B., and Rhoads, C. P.: Unpublished Observations.

(57) Rotter, W.: Über die histologischen Veränderungen des Dünndarms bei Ankylostomiasis, *Virchows Arch. f. path. Anat.,* 1931, **280**, 587.

(58) Suárez, R. M.: Clinical Aspects of Uncinariasis, *Puerto Rico J. Pub. Health and Trop. Med.,* 1933, **8**, 299.

(59) Cruz, W. O.: Pathogénie de l'anémie dans l'ankylostomose. Importance prépondérante d'une perturbation dans le métabolisme du fer dans l'organisme, *Compt. rend. Soc. de biol.,* 1932, **11**, 483.

(60) Boycott, A. E.: Anemia in Ankylostomiasis, *Brit. Med. J.*, 1907, **2**, 1318.

(61) Kobayashi, T.: Sur les phénomènes régénératifs des hématies dans les anémies de l'ankylostomiase, *Sang*, 1929, **3**, 129.

(62) Yang, C. S., and Keefer, C. S.: Liver and Iron in the Treatment of Secondary Anemia, *Nat. Med. J. China*, 1929, **15**, 721.

(63) Minot, G. R., and Castle, W. B.: The Adequate Treatment of Anemia, *Ann. Int. Med.*, 1931, **5**, 159.

(64) Cohn, E. J., Minot, G. R., Alles, G. A., and Salter, W. T.: The Nature of the Material in Liver Effective in Pernicious Anemia. II, *J. Biol. Chem.*, 1928, **77**, 325.

(65) Whipple, G. H., Robscheit-Robbins, F. S., and Walden, G. B.: Blood Regeneration in Severe Anemia. XXI. A Liver Fraction Potent in Anemia Due to Hemorrhage, *Am. J. Med. Sc.*, 1930, **179**, 628.

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HOOKWORM ANEMIA: ETIOLOGY AND TREATMENT WITH ESPECIAL REFERENCE TO IRON*

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INTRODUCTION

In regions of severe hookworm infection anemia has been regarded in a general way as synonymous with hookworm disease when the anemia is not due to other obvious causes. Although certain observations have been made which do not conform entirely to the simplicity of this idea, only in the past decade has the relationship of defects of nutrition to certain types of anemia been demonstrated in any quantitative manner. For this reason a resurvey of the problem of the anemia of hookworm disease seemed desirable in the light of recent discoveries concerning the etiology and treatment of hypochromic anemia not associated with hookworm infection. It was our good fortune to be able to carry out the observations described below under the auspices of the Rockefeller Foundation in 1931 in Puerto Rico. A preliminary report has already been published (1), and detailed descriptions of the observations will shortly be available (2).

Methods

Various studies were made on 83 patients, including children and adults, with anemia and hookworm infection. The patients, who were mostly from rural districts, were primarily selected because they had less than 50 per cent of the normal hemoglobin, had no complicating conditions, and had hookworm ova in the stools. They were observed either in various hospitals or as regular visitors to a special clinic established at the Presbyterian Hospital in San Juan. Permission has been given us to report briefly the results of field observations carried out upon 32 patients by Miss Celia Nuñez (3) at Cidra, a small agricultural community. Patients under observation in the hospitals, unless otherwise specified, were maintained on a basal diet resembling as closely as possible the usual food of the Puerto Rican peasants (see page 459). The presence of hookworms was assured by examination of the stools for ova or else all the worms expelled by efficient anthelmintics were collected and identified.

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* These observations were carried out in 1931 by the Commission of the Rockefeller Foundation for the Study of Anemia in Puerto Rico.

A systematic study of the morphological and physiological characteristics of the anemia was carried out, with particular attention to factors known to be of etiological significance in the production of types of hypochromic anemia not associated with hookworm disease. Particular attention was also given to the nature of the relationship of the hookworm to the anemia. The relative effectiveness of treatment of the anemia by removal of the parasites, by improvement of the diet, and by the administration of iron or liver extracts was determined. Hematopoietic effects were judged in hospital patients by daily observations of the course taken by the reticulocytes (4, 5), and by determinations of the red blood cell and hemoglobin values of the venous blood on alternate days or at appropriately longer intervals. In the field observations (3) the red blood cell and hemoglobin values were determined from capillary blood at approximately weekly intervals. Standardized apparatus was used. With the Sahli hemoglobinometer 100 per cent of hemoglobin was considered to be equivalent to 15.6 grams of hemoglobin per 100 cc. of blood.

Descriptive Observations

The blood picture. The outstanding features of the anemia were the reduction of average cell size and hemoglobin concentration. Except for the frequency of eosinophilia, the picture differed in no obvious way from the blood picture of types of hypochromic anemia not associated with hookworm infection encountered in Puerto Rico and elsewhere.

The bone marrow. A specimen of marrow was removed from the sternum at biopsy at various stages of the anemia in 15 patients, and the tissue subjected to histological study. The tissues were definitely more cellular than the normal marrow. The number of fat cells was decreased and vascularity was marked. Islands of young cells were present, representing a stage not far removed from hematopoietic vascular endothelium. A second variety of cell was seen, although not in large numbers, which was considered to belong to a primitive pluripotential cell type. The predominant cell was the normoblast. Large numbers of these cells were present, both in islands and diffusely scattered throughout the tissue. The reduced number of granulocytes present showed no disturbance of maturation. This bone marrow picture does not resemble the findings in severe anemia due to toxic or infectious conditions such as nephritis of severe grade. The bone marrow findings are in agreement with those found in other types of hypochromic anemia (6) and resemble somewhat the findings of others (7, 8) in the anemia of hookworm disease. The common genesis of these types of hypochromic anemia is thus further suggested.

The numerical relation of the hookworms to the degree of anemia. Smillie and Augustine (9), Schapiro (10), and others have observed that there is no consistent parallelism between the number of parasites and the degree of anemia. In our observations on 17 patients there was no observable correlation between the initial hemoglobin level and the total number of worms discharged. In 55 individuals the average number of ova in a centigram of feces, corrected for the consistency of the material, also showed no particular correlation with the initial hemoglobin value. These data are graphically presented in figure 1.

The diet. Suárez and his associates (11) have stated that the most important factor in the production of the anemias in Puerto Rico is the inadequate diet. A careful diet history of our 83 patients showed that the diets taken were very similar and could well be described in the words of Suárez (12). "The Porto Rican peasant breakfasts on coffee and a small loaf of pale white bread. His lunch consists of a large quantity of polished rice and kidney beans with scanty vegetables. His dinner is a stew of rice, chick-peas, pumpkin, and perhaps one or two tropical vegetables such as yautía. Once or twice a week he has the great good fortune to eat some salted codfish. This diet has been taken without variation since childhood. Many of these individuals have never had meat, eggs, or milk." The majority of the population in Puerto Rico affected by hookworm anemia is of the peasant class, serving as laborers on plantations for the raising of such "gold crops" as sugar, coffee, and tobacco. The concentration of the living quarters of the laborers makes small truck farming difficult, or it may even be expressly forbidden by the policy of the company. Poverty, local customs, and lack of refrigeration diminish to a minimum the consumption of meat and dairy products and result in the widespread use of dried beans and peas, polished rice, and salt codfish.

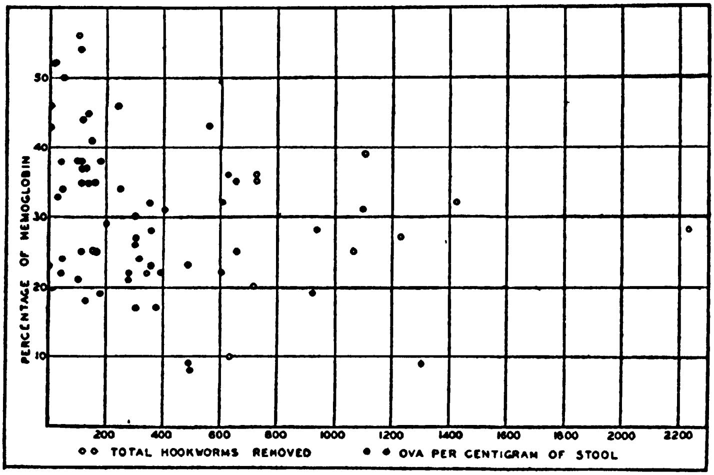

FIG. 1. Relationship between the original percentage of hemoglobin and the number of worms subsequently removed from 17 patients, and between the original percentage of hemoglobin and the average number of ova per centigram of stool of 55 patients.

The gastrointestinal pathology. Ashford and his colleagues (7) have pointed out the occasional loss of papillae of the tongue and the common occurrence of epigastric pain and tenderness, constipation, and diarrhea in sufferers from hookworm anemia. From our observations of the 54 patients upon whom gastric analyses were done, free hydrochloric acid was secreted in response to 50 cc. of 7 per cent alcohol by 30; only after the injection of 0.5 mgm. histamine by 11; and in 13 patients there was no secretion of free hydrochloric acid. Of the 47 patients adequately questioned, 8 gave a history or showed signs of glossitis, and 24 had had moderately severe diarrhea on certain occasions preceding the present illness.

EXPERIMENTAL OBSERVATIONS

The effect of removing the hookworms. The effectiveness of the removal of the hookworm burden in bringing about relief of the anemia has not been uniform in the experience of others (8, 13), although the elimination of the parasites has generally been considered necessary to a cure. Ashford, King, and Gutiérrez Igaravídez (7) concluded from their observations in Puerto Rico that the elimination of the hookworms would bring about rapid relief of the anemia in the majority of instances. The present observations exhibit a striking contrast to their conclusions. Twelve patients were kept in hospital upon the basal diet referred to above, and no form of therapy was given for the anemia. The parasites were removed with hexylresorcinol (14) and counted. From each of 4 patients over 1000 worms were recovered;

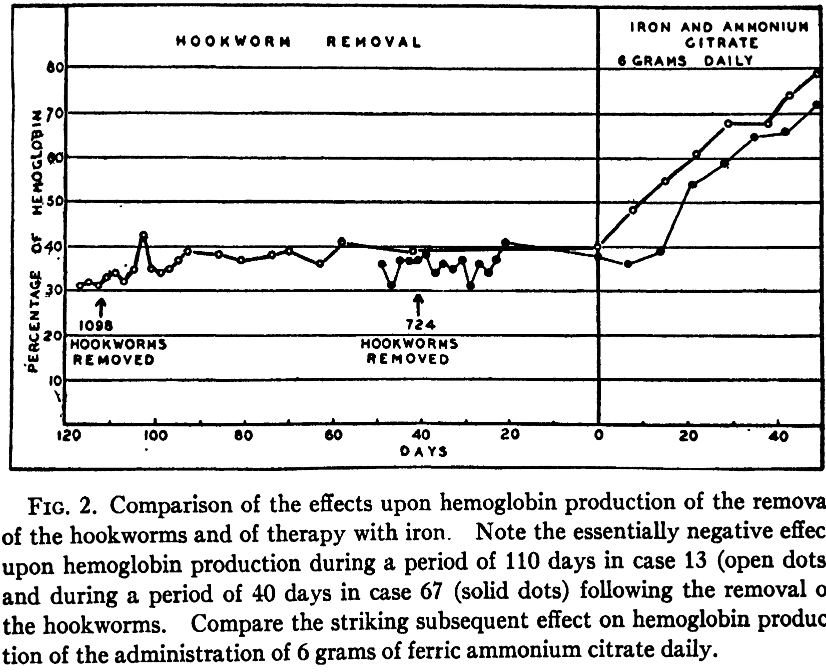

FIG. 2. Comparison of the effects upon hemoglobin production of the removal of the hookworms and of therapy with iron. Note the essentially negative effect upon hemoglobin production during a period of 110 days in case 13 (open dots) and during a period of 40 days in case 67 (solid dots) following the removal of the hookworms. Compare the striking subsequent effect on hemoglobin production of the administration of 6 grams of ferric ammonium citrate daily.

A. Hookworms removed
1. Diet unchanged ○ -- ○
2. High protein diet △ -- △
3. Diet unchanged □ -- □ and iron

B. Hookworms not removed
1. Diet unchanged ● -- ●
2. High protein diet ▲ -- ▲
3. Diet unchanged ■ -- ■ and iron

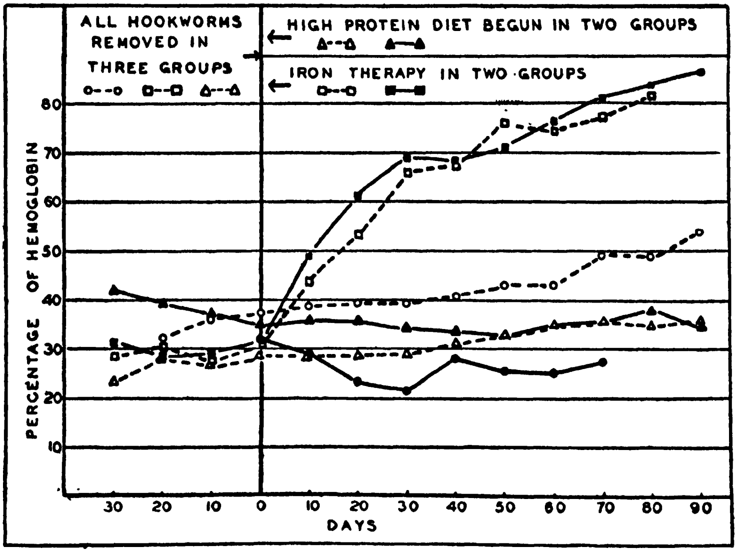

FIG. 3. Comparison of the effects of the removal of the parasites, of improved diets, and of therapy with iron. Results of observations in the field at Cidra on the average hemoglobin production in 6 groups of patients. The 32 patients were divided into two main groups, *A* and *B*, with three subgroups each, 1, 2, and 3, respectively. The 16 patients of group *A* (open symbols) had the hookworms removed before the beginning of the experimental period (vertical line in figure). The 16 patients of group *B* (solid symbols) received no treatment for hookworms. The patients of groups *A*-1 and *B*-1 (circles) had no change in diet. The patients of groups *A*-2 and *B*-2 (triangles) were given as an addition to their usual diet 300 gm. of meat and 1500 cc. of milk daily. The patients of groups *A*-3 and *B*-3 (squares) had no change in diet, but received daily 6 gm. of ferric ammonium citrate. Note the positive effects of iron in groups *A*-3 and *B*-3 in contrast to the negative or slight effects of removal of the hookworms or improvement of the diet.

from 8 others from 40 to 724. During periods of time as long as 28 days after the first treatment for hookworms, no significant effects upon reticulocyte, red blood cell, or hemoglobin values were produced. During 6 weeks the average gain in red blood cells of 7 patients was

0.49 million per cubic millimeter and in hemoglobin was 3 per cent. In figure 2 are shown the essentially negative effects upon the hemoglobin production of the removal of the hookworms in 2 patients of this series. Slight effects upon hemoglobin production and considerable increases of red blood cells during periods of as long as 90 days were noted in similar observations upon 6 patients in the field (see figure 3). The clinical improvement was, however, no greater than the slight effect upon hemoglobin production.

The best criterion of increased bone marrow activity is probably yielded by observations of the course of the reticulocytes (4) (5). For example, it is possible to demonstrate that in the anemia of malarial infection there is, as with other types of infection associated with fever, an inhibition of bone marrow function. Shortly after the abolition of the fever with quinine an increase of the reticulocytes in the peripheral circulation takes place. In none of the 12 patients with anemia associated with hookworm infection did the removal of the parasites have any effect upon reticulocyte production. In figure 4 is illustrated the contrast in this respect between the typical effects of removing the parasites in a patient with malaria and in a patient with hookworm infection. Both patients were severely anemic. The absence of any increase of reticulocytes after the removal of the hookworms affords no evidence for a dominant inhibitory effect of the parasites on the bone marrow function. The fact that removal of the hookworms produced slight effects upon hemoglobin production and considerable increases of red blood cells during prolonged periods is not necessarily evidence of increased activity on the part of the bone marrow. Precisely such a result would occur if the amount of blood loss were diminished.

The effect of hematopoietic substances on blood production without removal of the hookworms Although Ashford, King, and Igaravídez (7) did not consider therapy with iron of any particular importance compared to the elimination of the hookworms in the relief of the anemia in Puerto Rico, a careful scrutiny of the protocols of their observations suggests that the iron administered, in many instances, was of importance in bringing about the rapid improvement observed. This interpretation finds support in the observations of others (8) (13, 15, 16), as well as in our experience. The effect was observed of the daily oral administration of 6 grams of ferric ammonium citrate to each of 27 patients whose hookworm burden had not been removed. No changes in diet were made. Increases of reticulocytes and of red blood cells and hemoglobin promptly appeared. By plotting upon

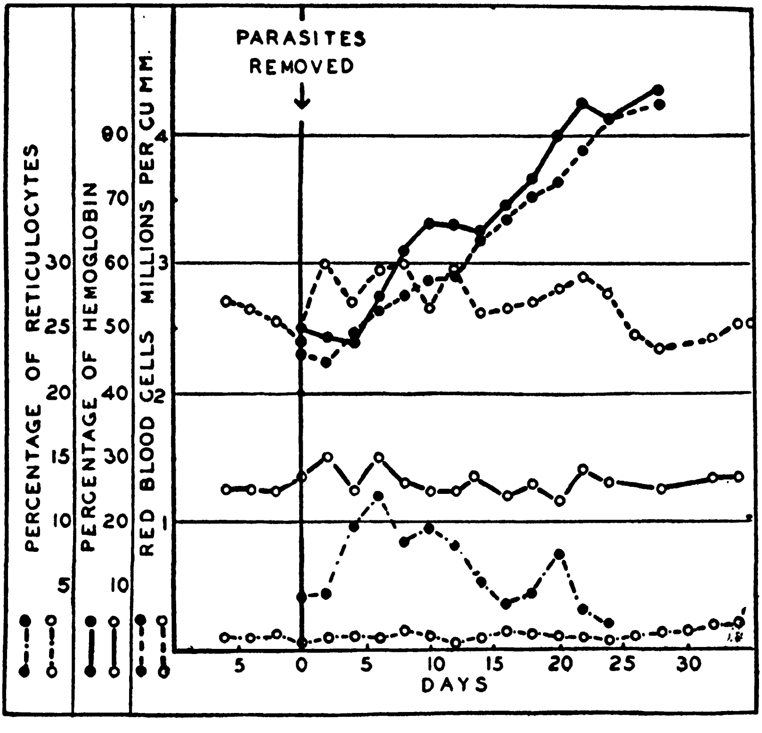

Fig. 4. Comparison of the effects upon hematopoiesis of the removal of the parasites in a patient with anemia associated with malarial infection (solid dots), and in a patient with anemia associated with hookworm infection (open dots). After abolition of the parasites, note the increase of reticulocytes in malaria, and the absence of such effect in hookworm disease. Note also the rapid improvement of the blood values in the anemia of malaria, and the absence of improvement in the anemia of hookworm disease.

coordinate paper, against days, the actual determinations of the hemoglobin percentages made in each case, the values for each patient at 10-day intervals were read off and are shown in table 1. The average increase of hemoglobin during the first 30 days in these 27 patients

TABLE 1

Effect upon Hemoglobin Production of the Daily Administration of 6 Grams of Ferric Ammonium Citrate

Hookworms not removed

Case number	Hemoglobin percentages at 10-day intervals						
	0 days	10 days	20 days	30 days	40 days	50 days	60 days
4	35	38	48	55	63	68	
5	44	48	58	68	71		
7	44	55	64	70	76	83	85
8	34	40	57	63	72	75	83
11	21	40	52	57	61	66	70
16	22	44	54	64	68	70	72
23	25	37	46	53			
24	44	57	66	76			
34	43	53					
37	18	34	41	50	49	52	
38	42	49	57	64	72	77	
39	17	20	26	32	35	41	44
41	36	42	52	56			
42	25	33	43	55	61	67	69
43	35	40	42				
46	41	50	59	65	69	71	73
47	26	47	56				
48	30	40					
56	40	50	62	68	72		
66	40	51	58	62	70	78	80
69	35	45	52	59	64	68	70
71	41	50	55				
72	22	30	41	50			
73	36	50					
74	34	41	51	56			
76	45	58	63				
80	41	52	66	62	70		
Averages....	34	44	53	59	65	68	72
Hookworms previously removed							
13	40	50	54	68	70	80	
52	27	38	47				
59	33	47	59				
60	22	37					
63	36	48					
67	38	37	52	60	66	71	
Averages....	33	43	53	64	68	76	

was 25 per cent. In 6 patients who had previously had their hookworm burden removed, the administration of similar doses of iron produced an increase of 31 per cent of hemoglobin during the first 30 days (see table 1). Similar observations were made in the course of the field observations. In 5 patients, whose hookworm burden was removed, during the first 30 days of treatment with iron the average gain in hemoglobin (35 per cent) was slightly less than the average gain in hemoglobin (38 per cent) of 7 patients who had received no treatment for the parasite. During the first 60 days of therapy with iron each group gained 44 per cent of hemoglobin (see figure 3). The clinical improvement corresponded to the rapid increase in hemoglobin whether the parasites were present or not.

Cohn, Minot, and their collaborators (17) prepared certain fractions of liver in developing an effective substance for the treatment of pernicious anemia. Certain commercial preparations of these fractions were administered to suitable patients with hookworm anemia under controlled conditions in the hospital. Suárez (18) has described the effectiveness of an aqueous extract of liver in the treatment of the anemia of hookworm disease. The amount of this material, Liver Extract E–29,* derived from 340 grams of liver, was given daily to each of 15 of our patients. The average increase in hemoglobin in 30 days was only 13 per cent compared with 25 per cent in the group of patients receiving 6 grams of ferric ammonium citrate daily. The sub-fraction of liver which is insoluble in 70 per cent alcohol (19) is commercially available in dry form as Liver Extract no. 55, except that in this particular preparation approximately 0.5 gram of ferric ammonium citrate is added to the amount of extract derived from 100 grams of liver. This material, prepared without the added iron,† was administered to 7 patients in daily doses of the material derived from 400 grams of liver. Moderate effects upon hematopoiesis were observed in 4 patients and no significant effects in 3 others. After the discontinuation of this liver extract, a daily dose of 2 grams of ferric ammonium citrate was given. This dose corresponds to the amount of iron added in the commercial preparation of Liver Extract no. 55 to the amount of liver extract derived from 400 grams of liver. Secondary increases of reticulocytes were observed in all instances, indicating the greater potency of the iron salt. Liver Extract no. 343,* represents a commercial preparation of the sub-fraction of liver which is soluble in 70 per cent but insoluble in 95 per cent alcohol by volume. Orally administered in daily doses of the amount derived from 300 grams of liver (20) and intramuscularly administered in daily doses derived from 10 grams of liver (21), it is highly potent in pernicious anemia. Such dosages, however, were quite without effect in the hypochromic anemia of hookworm disease when tested upon 11 patients.

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* Kindly supplied by Valentine's Meat Juice Company.

† Kindly supplied without added iron by Eli Lilly and Company.

The basal diet of 8 patients in the hospital was increased by the daily addition of 1500 cc. of milk and 300 grams of lean beef, which would presumably yield an excellent supply of animal protein, would provide certain vitamins, and would serve as sources of organic iron and calcium. Within periods of time varying from 14 to 18 days, no significant effect upon reticulocyte production, hemoglobin, or red blood cell values was produced. The negative effect of the administration of the improved diet, contrasted with the positive effect of iron upon reticulocyte and hemoglobin production in 2 of these patients, is graphically presented in figure 5. Similar negative results from improved diets during longer periods were observed under field conditions (see figure 3).

DISCUSSION

It is clear that in the anemia of hookworm disease the morphology of the blood picture and of the bone marrow, as well as the response to hematopoietic materials, does not differ from that of types of hypochromic anemia unassociated with hookworm infection. It is, of course, conceivable but unlikely that such similarities could be presented by an anemia produced in some incomprehensible way solely by the parasites. However, the effect of the hookworms could understandably be that of a hemolytic process (22), of a toxic process inhibiting the bone marrow (23), or of a process leading to blood loss (24). Clearly the morphological characteristics of the blood picture and bone marrow are not those of a hemolytic process, such as is illustrated by chronic hemolytic jaundice, in which increased numbers of reticulocytes and increased serum color are constantly present.

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* Kindly supplied by Eli Lilly and Company.

Fülleborn (23) states that the hookworm secretes a substance which has a toxic effect on the bone marrow. If the presence of the hookworms has a dominant effect in depressing bone marrow activity, the removal of the parasites should produce strikingly increased blood regeneration, and the presence of the parasites should inhibit the influence of agents capable of promoting blood formation. The observations reported above yield no evidence for either of these two effects.

It remains to consider chronic blood loss which, in animals and in man (24), may result in hypochromic anemia with exactly the characteristics which we have observed in the anemia of hookworm disease. This explanation of the influence of the hookworms correlates perfectly with known facts and readily explains the resemblances of the blood picture and bone marrow to those of other types of hypochromic anemia not associated with hookworms. From direct observations of the hookworms of dogs, Wells (25) estimated that in 24 hours 100 hookworms removed 84 cc. of blood. Bass (26) states that tests of the feces of patients for occult blood are nearly always positive even in mild

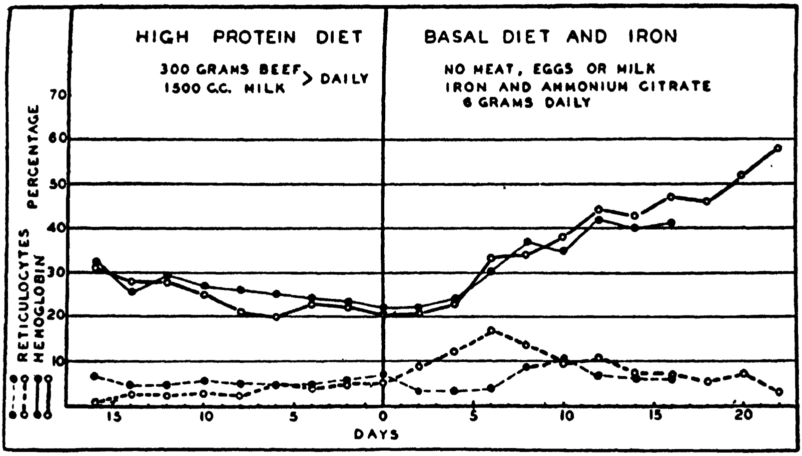

FIG. 5. The negative effect upon hemoglobin and reticulocyte production of the daily addition of 300 grams of meat and of 1500 cc. of milk to the basal diet, contrasted with the positive effect of the daily addition of 6 grams of ferric ammonium citrate to the basal diet. The results are shown in 2 patients (open dots and solid dots). No therapy for hookworms was given.

or moderate infections with hookworms. We observed hematopoietic effects from injected erythrocytes in 2 patients. Blood loss alone, however, cannot be the whole explanation of the production of the anemia, since there is no consistent parallellism between the number of parasites and the degree of the anemia in our experience and in that of others (9, 10). It is, therefore, logical to look for the influence of other factors common in producing hypochromic anemia not associated with hookworm disease.

The work of several observers (27, 28, 29) demonstrates that hypochromic anemia in the temperate zone is due mainly to a lack of available iron in the body, resulting in a failure of the bone marrow to produce adequate amounts of red blood cells and hemoglobin. It has been demonstrated that the amount of hematopoietic substances, including iron, may become reduced in the body from chronic blood loss (24), from a lowered iron intake in the diet (30), or because absorption of iron from the intestinal tract is inadequate (31) (32). A careful study of the diet history of 56 of our patients bears out the belief of Suárez (11) that there is a relationship between defective nutrition and the anemia of hookworm disease in Puerto Rico. The exact relationship of the dietary defects is, however, not entirely clear since, in Puerto Rican diets, there are apparently good sources of iron due to the presence of large amounts of peas and beans. On the other hand, the typical diets are decidedly deficient in other food substances of importance in the production of hemoglobin (33) and are generally deficient in animal protein, minerals, and in vitamins A, B, and D.

The relationship of altered gastrointestinal function to hypochromic anemia has been emphasized by the work of various authors. Glossitis (34, 35), gastric anacidity (31), and diarrhea (32) are commonly associated with cases of hypochromic anemia and have been shown to have an etiological bearing. Ashford and his colleagues (7) mentioned loss of papillae of the tongue and the common occurrence of digestive symptoms in sufferers from hookworm anemia. Hypoacidity was present in about 20 per cent, and anacidity in about 24 per cent of our patients. Of 47 patients, 8 gave a history or showed signs of glossitis and 24 had had moderately severe diarrhea on certain occasions preceding the present illness. Rotter (36) has described morphological changes in the intestinal tract of patients with hookworm disease.

In the light of observations of McCarrison (37) and others (38) (39), it is probable that defective nutrition may be responsible for morphological changes in the intestinal tract, productive not only of symptoms but also of difficulty with the assimilation of elements directly essential for hematopoiesis. Such a supposition is clearly necessary in comprehending the etiology of certain cases of hypochromic anemia not associated with hookworm infection, for the oral dosage of iron salts needed to produce a given hematopoietic effect varies as does the oral dosage of liver extract in different patients with pernicious anemia or sprue. In varying degrees in different patients the factors of food deficiency, gastrointestinal changes, and blood loss may obviously contribute to the production of the type of hypochromic anemia associated with hookworm infection no less than when hookworms are not present. No evidence was produced by our studies for any effect of the hookworms other than that of blood loss.

The observation was repeatedly made that the removal of the parasites resulted in little improvement of the hemoglobin level or of the clinical condition of the patients, sometimes over periods of as long as 90 days. On the other hand, without removal of the parasites, prompt improvement of the blood values and of the clinical condition invariably resulted from the administration of iron salts without change in the diet. The importance of measures directed against the parasite is not to be deprecated, but the possibility of a quick return to better health and economic usefulness should aid in securing cooperation in such prophylactic measures as the wearing of shoes, the erection of adequate latrines, and the use of better foods.

Since the presence of the worms without anemia is not necessarily detrimental to health, it should be possible to employ iron salts as a prophylactic, either alone or preferably in combination with anthelmintics. The cost of the treatment necessary to relieve the anemia is astonishingly low. The daily dosage of ferric ammonium citrate for adults should be at least 4, preferably 6 grams a day for maximum effects. The full amounts should not be given at first, and later only in divided doses after meals. In children smaller doses are desirable. Undoubtedly the prophylactic doses of iron need not be so large or could be administered at greater intervals of time. Observations on this point should be of great practical value. Other preparations of iron are satisfactory for routine use in correspondingly large dosage. As with other types of hypochromic anemia associated with changes in the gastrointestinal tract, it is probably essential in many instances to continue the administration of iron after the patient has been returned to health, even though the parasites have been eliminated. Since the effect of the liver fractions tested was found to be inferior to that of iron salts, and their cost far greater, liver extracts offer no advantages over iron in the treatment of this type of hypochromic anemia. As the symptomatology of hookworm infection is predominantly that of anemia, the primary importance and simplicity of dealing directly with the anemia should be manifest.

CONCLUSIONS

1. The hypochromic anemia of hookworm disease in Puerto Rico is apparently caused by a combination of blood loss, dietary deficiency, and gastrointestinal changes probably resulting from defective nutrition.
2. The removal of the hookworms without other therapy had very little effect upon anemia or clinical condition.
3. With or without removal of the hookworms the administration of large doses of iron produced rapid improvement of blood values and clinical conditions.
4. The advantages of *direct* treatment of the anemia with iron are indicated.

BIBLIOGRAPHY

1. Rhoads, C. P., and Castle, W. B.
1932. Observations on the etiology and treatment of the anemia of hookworm disease in Porto Rico. Jour. Clin. Inv., *11*, 809.
2. Rhoads, C. P., Castle, W. B., Payne, G. C., and Lawson, H. A.
Observations on the etiology and treatment of anemia associated with hookworm infection in Puerto Rico. Medicine. In press.
3. Nuñez, C.
A study of the relative effects of removal of hookworms, of dietary therapy, and of iron in medicinal form upon the anemia of hookworm infestation. In press.
4. Minot, G. R., Murphy, W. P., and Stetson, R. P.
1928. The response of the reticulocytes to liver therapy: Particularly in pernicious anemia. Amer. Jour. Med. Sc., *175*, 581.

5. Minot, G. R., and Heath, C. W.
   1932. The response of the reticulocytes to iron. Amer. Jour. Med. Sc., *183*, 170.

6. Dameshek, W.
   1931. Primary hypochromic anemia (erythro-normoblastic anemia). Amer. Jour. Med. Sc., *182*, 520.

7. Ashford, B. K., King, W. W., and Gutiérrez Igaravídez, P.
   1904. Report of the study and treatment of "anemia" in Porto Rico. Bur. Print. and Supplies, San Juan.

8. Day, H. B., and Ferguson, A. R.
   1914. The treatment of ankylostoma anemia. Lancet, *2*, 82.

9. Smillie, W. G., and Augustine, D. L.
   1926. Hookworm infestation. Effect of varying intensities on the physical condition of school children. Amer. Jour. Dis. Child., *31*, 151.

10. Schapiro, L.
    1930. Hookworm infestation in an Indian (Guaimi) and non-Indian population of Panama. Amer. Jour. Trop. Med., *10*, 365.

11. Suárez, R. M., Morales Otero, P., and Rodrígues Molina, R.
    1922. Estudio de dos casos de anemia. Bol. Asoc. méd. de Puerto Rico, *22*, 5.

12. Suárez, R. M.
    1930. Puerto Rico and some of its health problems. Bol. Asoc. méd. de Puerto Rico, *22*, 197.

13. Boycott, A. E.
    1907. Anemia in ankylostomiasis. Brit. Med. Jour., *2*, 1318.

14. Lamson, P. D., Brown, H. W., Robbins, B. H., and Ward, C. B.
    1931. Field treatments of ascariasis, ancylostomiasis and trichuriasis with hexylresorcinol. Amer. Jour. Hyg., *13*, 803.

15. Kobayashi, T.
    1929. Sur les phénomènes régénératifs des hématies dans les anémies de l'ankylostomiase. Sang, *3*, 129.

16. Yang, C. S., and Keefer, C. S.
    1929. Liver and iron in the treatment of secondary anemia. Nat. Med. Jour. China, *15*, 721.

17. Cohn, E. J., Minot, G. R., Alles, G. A., and Salter, W. T.
    1928. The nature of the material in liver effective in pernicious anemia, II. Jour. Biol. Chem., *77*, 325.

18. Suárez, R. M.
    1931. El tratamiento del esprú y el uso del extracto acuoso de hígado en su anemia. Bol. Asoc. méd. de Puerto Rico, *23*, 74.

19. Whipple, G. H., Robscheit-Robbins, F. S., and Walden, G. B.
    1930. Blood regeneration in severe anemia. XXI. A liver fraction potent in anemia due to hemorrhage. Amer. Jour. Med. Sc., *179*, 628.

20. Minot, G. R., and Castle, W. B.
1931. The adequate treatment of anemia. Ann. Int. Med., *5*, 159.

21. Strauss, M. B., Taylor, F. H. L., and Castle, W. B.
1931. Intramuscular use of liver extract. Jour. Amer. Med. Assn., *97*, 313.

22. Whipple, G. H.
1909. The presence of a weak hemolysin in the hookworm and its relation to the anemia of uncinariasis. Jour. Exp. Med., *11*, 331.

23. Fülleborn, F.
1929. Epidemiological observations on hookworm infection. Brit. Med. Jour., *1*, 755.

24. Drinker, C. K.
1920. Diseases of the blood. The pathological physiology of blood cell formation and blood cell destruction. The Oxford Medicine, Oxford University Press, New York, *2*, 509.

25. Wells, H. S.
1931. Observations on the blood sucking activities of the hookworm, *Ancylostoma caninum*. Jour. Parasitol., *17*, 167.

26. Bass, C. C.
1929. Hookworm disease. Practice of Medicine, Tice, W. F. Prior Co., Hagerstown, Md., *5*, 79.

27. Meulengracht, E.
1923. Large doses of iron in the different kinds of anaemia in a medical department. Acta med. Scandinav., *58*, 594.

28. Witts, L. J.
1931. The therapeutic uses of iron. Proc. Roy. Soc. Med., *24*, 7.

29. Heath, C. W., Strauss, M. B., and Castle, W. B.
1932. Quantitative aspects of iron deficiency in hypochromic anemia (the parenteral administration of iron). Jour. Clin. Inv., *11*, 1293.

30. Minot, G. R.
1929. Some fundamental clinical aspects of deficiencies. Ann. Int. Med., *3*, 216.

31. Mettier, S. R., and Minot, G. R.
1921. The effect of iron on blood formation as influenced by changing the acidity of the gastroduodenal contents in certain cases of anemia. Amer. Jour. Med. Sc., *181*, 25.

32. Keefer, C. S., Huang, K. K., and Yang, C. S.
1929. The importance of undernutrition in the production of anemia associated with chronic dysentery and tuberculosis of the intestine. Nat. Med. Jour. China, *15*, 743.

33. Robscheit-Robbins, F. S.
1929. The regeneration of hemoglobin and erythrocytes. Physiol. Rev., *9*, 666.

34. Witts, L. J.
    1931. The syndrome of glossitis, dysphagia and anemia. Guy's Hosp. Rep., *11*, 193.

35. Hutter, A. M., Middleton, W. S., and Steenbock, H.
    1933. Vitamin B deficiency and the atrophic tongue. Jour. Amer. Med. Assn., *101*, 1305.

36. Rotter, W.
    1931. Über die histologischen Veränderungen des Dünndarms bei Ankylostomiasis. Virchows Arch. f. path. Anat., *280*, 587.

37. McCarrison, R.
    1921. Studies in deficiency disease. Oxford Medical Publications, Henry Frowde and Hodder and Stoughton, London.

38. Tilden, E. B., and Miller, E. G.
    1930. The response of the monkey to withdrawal of vitamin A from the diet. Jour. Nutrition, *3*, 121.

39. Rhoads, C. P., and Miller, D. K.
    1933. The production in dogs of chronic black tongue with anemia. Jour. Exp. Med., *58*, 585.

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THE MATERNAL TRANSMISSION OF VACCINIAL IMMUNITY IN SWINE

II. THE DURATION OF ACTIVE IMMUNITY IN THE SOW AND OF PASSIVE IMMUNITY IN THE YOUNG

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Observations on the maternal transmission of immunity to a filterable virus, namely vaccinia virus in swine, were reported in a preceding paper.[1] It was found that pigs which had received colostrum from their vaccinated dams were generally refractory to vaccination during the early days of life, whereas their litter mates which had been raised without colostrum regularly responded to vaccination with a typical cutaneous reaction. It was concluded that the porcine placenta is impermeable to any appreciable amount of the protective substance elicited as the result of vaccination and that the colostrum functions as the vehicle for its transport to the young.

The present report is concerned with 3 additional phases of the transmission of vaccinial immunity in swine, (1) the duration of maternal transfer; (2) the duration of immunity acquired by the suckling young, and (3) the effect of early vaccination on the persistence of this immunity.

The Duration of Maternal Transfer

Two immune sows which had originally been vaccinated in the late fall of 1930 were followed through 6 successive farrowings with the view of determining how long they would continue to transmit protective substances to their nursing young.

Suckling pigs from each of the 6 litters farrowed by the 2 sows were vaccinated during the 1st or 2nd week of life. The test pigs were chosen at random and were believed to be representative of the several litters. The vaccinia virus[2] was rubbed into parallel scratches made in the skin over the inner surface of the flank. The vaccinated pigs were subsequently kept under observation for a period of 10 to 14 days and during this period they continued to nurse their respective dams.

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[1] Nelson, J. B., *J. Exp. Med.*, 1932, **56**, 835.

The results of the vaccination tests on the suckling young from the 2 immune sows, together with the size of the litters and the dates of farrowing are presented in Table I. The pigs from each pair of the 6 litters were uniformly refractory to vaccinia, there being no response to the cutaneously introduced virus in any case. In addition to the young which were tested during the 1st or 2nd week of life a total of 13 pigs, from various litters, were vaccinated at a later time. These pigs likewise failed to show a cutaneous reaction. The suckling young of

TABLE I

The Reaction of the Suckling Young from 6 Successive Litters to Vaccination

Litter No.	Sow No.*	Date of farrowing	No. of pigs	No. of suckling pigs vaccinated	No. of reactors
1	1	Apr. 6, 1931	7	3	0
	2	May 1, 1931	9	3	0
2	1	Oct. 1, 1931	6	3	0
	2	Oct. 18, 1931	8	4	0
3	1	Mar. 20, 1932	6	3	0
	2	Apr. 14, 1932	12	3	0
4	1	Sept. 9, 1932	6	3	0
	2	Oct. 10, 1932	12	4	0
5	1	Mar. 8, 1933	4	2	0
	2	Apr. 28, 1933	10	4	0
6	1	Sept. 5, 1933	7	4	0
	2	Oct. 11, 1933	6	3	0

* The sows were vaccinated in the late fall of 1930.

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[2] The vaccinia virus employed in vaccination was obtained from the Laboratories of the New York City Department of Health through the courtesy of Dr. W. H. Park.

non-vaccinated sows, which were tested at intervals as a control on the potency of the vaccinia virus, invariably developed vesicles at the site of vaccination.

The Duration of the Immunity Acquired by the Suckling Young

With a few exceptions the pigs from the 2 vaccinated sows were allowed to nurse until about the 6th week when they were weaned. In order to determine the approximate duration of the immunity acquired by suckling, representatives from most of the litters were removed at different age intervals and vaccinated.

The results of this experiment are summarized in Table II. Thirteen suckling pigs vaccinated 4 to 5 weeks after birth showed no reaction. Three of 13 weaned pigs vaccinated 8 to 9 weeks after birth likewise failed to react. Ten of the individuals in this group, however, developed small papules. These were generally discrete, showed little or no color, and usually began to retrogress on the 2nd or 3rd day after they appeared. There was little or no scab formation. This is referred to as a papular reaction in Table II and is regarded as suggestive of a declining immunity. Five of the 19 pigs vaccinated after 12 to 13 weeks also showed a papular reaction. In the remaining 14 pigs of this group vaccination was attended by vesicle formation. The vesicles were usuallv somewhat smaller than those in fully susceptible control pigs and were more commonly discrete. It is believed, however, that the reaction is sufficiently typical to be indicative of a return to nearly complete susceptibility.

TABLE II

The Duration of Vaccinial Immunity in the Suckling Young of Immune Sows

	4–5 wks.	8–9 wks.	12–13 wks.
No. tested	13	13	19
No. of reactors	0	10 papular	14 vesicular 5 papular

The Effect of Early Vaccination on the Persistence of Suckling Immunity

It was considered of interest to determine whether or not the vaccinia virus which was introduced cutaneously in the nursing pigs during the first few days of life exerted any immunizing effect in the absence of a visible local reaction. Was the transient immunity acquired by suckling converted thereby into one of long duration? To answer this question suckling pigs which had been vaccinated, without take, shortly after birth were revaccinated 12 to 13 weeks later and their response compared with that in litter mates which had also nursed but had not been previously vaccinated.

The results of the tests on 9 pairs of pigs from 6 different litters are presented in Table III. In eight cases the paired pigs showed practically identical reactions. In one instance only, Pair 5, was a difference in the degree of susceptibility indicated. The previously vaccinated pig in this case showed a papular reaction while its unvaccinated litter mate responded with the formation of vesicles.

TABLE III

The Response of Vaccinated and Unvaccinated Litter Mates to Vaccination during the 12th to 13th Week of Life

No.	Type of reaction	
	Vaccinated young	Unvaccinated young
1	Vesicular	Vesicular
2	"	"
3	Papular	Papular
4	Vesicular	Vesicular
5	Papular	"
6	Vesicular	"
7	"	"
8	"	"
9	Papular	Papular

DISCUSSION

Since there is no reason to believe that the suckling pigs which were tested were different in any respect from their unvaccinated litter mates, it can be assumed that their immune dams produced and transported in their colostrum, during the 3 year period of observation, a sufficient amount of protective substance to afford immunity in the one case to 37 and the other to 57 young. These figures represent the total number of pigs farrowed by each of the 2 sows, respectively.

It appears evident that the immune mechanism of the vaccinated sow is able to compensate repeatedly for the amount of protective substance which is lost with each parturition. The actual concentration which is lost in this way must be considerable since protection may be afforded to as many as 12 individuals at the termination of a single pregnancy.

The immunity acquired by the nursing pigs was clearly passive. In most instances complete protection was afforded only during the first 4 or 5 weeks after birth, at least in so far as the concentration of virus employed in vaccination was concerned. The immunity began to decline during the 2nd month and by the end of the 3rd month approximately 75 per cent of the pigs were again susceptible. There was no indication that the decline in protection was any more rapid with the pigs of the 6th litters than with those of the earlier ones. The duration of the suckling immunity was not significantly prolonged by the virus which was cutaneously introduced, without reaction, during the early days of life.

SUMMARY

The protective substances produced by vaccinia virus in swine are transmitted repeatedly to the young by way of the colostrum of the sow. In 2 instances suckling immunity was demonstrable in the young of 6 successive farrowings which numbered 37 and 57 individuals, respectively.

The immunity acquired by suckling began to decline during the 2nd month and was practically negligible by the end of the 3rd month. Vaccinia virus introduced cutaneously during the first few days of life in the passively protected pigs exerted little or no immunizing effect.

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THE DIFFERENTIATION OF TRYPSINS BY MEANS OF THE ANAPHYLACTIC TEST

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Kunitz and Northrop (1) have recently reported the crystallization of chymotrypsinogen and chymotrypsin and have shown that the latter differs from the trypsin that they had studied previously. The present work was undertaken to determine whether these substances could be differentiated by immunological reactions. Trypsin from the pig and cow as well as chymotrypsinogen and chymotrypsin from the cow were used.

The studies on the antigenic nature of enzymes made before these substances were purified by crystallization are all open to question, for it is impossible to determine whether the reactions obtained were due to the enzymes as such or to the proteins with which they were associated. Northrop (2) found that the serum of a rabbit immunized to crystalline pepsin precipitated this enzyme when it was diluted to 1:2000. This immune serum had a greater antipeptic action than did the serum from a normal rabbit or from a rabbit immunized to the pepsin denatured by heat. Sumner and Kirk (3) have shown that their crystalline urease was antigenic, for they produced in rabbits an antiurease that *in vitro* and *in vivo* neutralized the action of the enzyme. Kirk (4) was also able to cause the production of a precipitin that he has used in purifying urease. Kirk and Sumner (5) were unable to differentiate by the precipitin or the antiurease reaction the urease obtained from the soy or jack bean.

The essential part of this work, namely the preparation of the enzyme solutions, was done by Drs. Northrop and Kunitz. The enzymes have been recrystallized at least five times and were kept in concentrated, slightly acid solution in the cold. Fresh dilutions of known concentration were prepared for each test.

The anaphylactic test rather than the less sensitive precipitin reaction was used in the major portion of the tests for differentiation since the enzymes may be very closely related.

Sensitization of Guinea Pigs—Female guinea pigs weighing about 125 gm. were given subcutaneous injections of 0.5 cc. of either 0.5 or 1 per cent solution of the enzymes. Injection of chymotrypsinogen produced no visible effect, but the animals receiving the trypsin and the chymotrypsin showed necrotic areas at the site of inoculation and several died. It was our impression that the chymotrypsin was more toxic than the trypsin.

TABLE I

Results of Tests for Sensitiveness of Uteri of Guinea Pigs Receiving Pig Trypsin

In this and in Tables II to IV L and R indicate left and right horns of the uterus. A = first test; B, second; etc. Figures are the concentration of the enzymes in the bath in mg. of protein per ml. + or – indicates contraction or failure to contract.

Guinea pig No.	Reaction to		
	Pig trypsin	Beef trypsin	Chymotrypsin
1	RB 10^{-4} –	RA 10^{-3} –	
	RC 10^{-3} +		
	LA 10^{-4} –		
	LB 10^{-3} +		
2	LB 10^{-3} +	LA 10^{-3} –	
	RB 10^{-3} +		RA 10^{-3} –

One guinea pig was not sensitive.

Tests for Sensitization—Between 15 and 20 days after the first injection, the uteri of these guinea pigs were tested by means of the Dale technique (6). Two baths were used and the two horns of the uterus were tested, one after the other. The capacity of each bath was 75 cc., and 0.75 cc. of the enzyme solution was added for each test.

The results of the tests are given in Tables I to IV. Not all of the animals were sensitized, and in some cases there were cross-reactions, particularly between the chymotrypsin and chymotrypsinogen. The results were, however, sufficiently clear cut to show that all four of these enzymes can be differentiated by this reaction.

Tests for Precipitin—The undiluted serum from a rabbit that had received repeated injections of chymotrypsin was layered under dilutions of this enzyme. In 1 per cent and 0.5 per cent solutions of the enzyme there were definite ring formations, and after the serum and enzyme were mixed, definite turbidity and precipitate. In solutions of 0.25 and 0.125 per cent there were questionable rings, but after mixing no turbidity nor precipitate could be detected. The sera of two rabbits that had received

TABLE II

Results of Tests for Sensitiveness of Uteri of Guinea Pigs Receiving Beef Trypsin

See Table I for explanation of symbols.

Guinea pig No.	Reaction to			
	Beef trypsin	Pig trypsin	Chymotrypsin	Chymotrypsinogen
3	LA 10^{-5} −			
	LB 10^{-4} +			
	RB 10^{-3} +	RA 10^{-3} −		
4	LB 10^{-3} +	LA 10^{-3} −		
	RA 10^{-4} +	$\mathrm{R}^{w*}\mathrm{B}$ 10^{-2} ?		
5	$\mathrm{L}^{w}\mathrm{B}$ 10^{-3} ?	LA 10^{-2} ?		
	RB 10^{-3} +		RA 10^{-2} −	
6	LC 10^{-4} +	LA 10^{-3} −		LB 10^{-3} −
	RB 10^{-4} +		RA 10^{-3} −	
7	LB 10^{-4} +		LA 10^{-2} −	
	RB 10^{-4} +	RA 10^{-3} −		
8	LB 10^{-4} +		LA 10^{-3} −	
	RB 10^{-4} +			RA 10^{-2} −
9	LC 10^{-4} +		LA 10^{-2} ?	
			$\mathrm{L}^{w}\mathrm{B}$ 10^{-3} −	
	RB 10^{-4} +		RA 10^{-3} −	
10	LB 10^{-3} +			LA 10^{-3} −
	RB 10^{-3} +		RA 10^{-3} −	
11	LB 10^{-3} +			LA 10^{-2} −
	RB 10^{-3} +		RA 10^{-3} −	

Three guinea pigs were not sensitive to the trypsin solution.

[*] w indicates that the horn was washed and allowed to relax before testing.

TABLE III

Results of Tests for Sensitiveness of Uteri of Guinea Pigs Receiving Chymotrypsinogen

See Table I for explanation of symbols.

Guinea pig No.	Reaction to		
	Chymotrypsinogen	Chymotrypsin	Beef trypsin
12	LB 10^{-3} +	LA 10^{-3} −	
	RB 10^{-3} ±		RA 10^{-3} −
13	LB 10^{-3} ?	LA 10^{-2} −	
	RA 10^{-3} +		
14	LB 10^{-4} +	LA 10^{-3} −	
	RB 10^{-3} +	RA 10^{-2} −	
15	LA 10^{-5} −		
	LB 10^{-4} +		
	RB 10^{-3} +	RA 10^{-3} −	
16	LB 10^{-3} +	LA 10^{-3} −	
		RA 10^{-2} −	
17	LB 10^{-3} +	LA 10^{-3} −	
	R^{w}B 10^{-3} ±	RA 10^{-2} −	
18	LA 10^{-4} +		
	RD 10^{-4} −	RA 10^{-4} −	
	RE 10^{-3} +	RB 10^{-3} −	
		RC 10^{-2} −	
19	L^{w}B 10^{-3} +	LA 10^{-3} −	

Two guinea pigs were not sensitive and four gave reactions with chymotrypsin as well as chymotrypsinogen.

TABLE IV

Results of Tests for Sensitiveness of Uteri of Guinea Pigs Receiving Chymotrypsin

See Table I for explanation of symbols.

Guinea pig No.	Reaction to	
	Chymotrypsin	Chymotrypsinogen
20	LA 10^{-3} +	
	RC 10^{-2} +	RA 10^{-3} −
		RB 10^{-3} −
21	LA 10^{-3} +	
		RA 10^{-2} −

Two guinea pigs were not sensitive.

repeated injections of chymotrypsinogen failed to cause rings or precipitate when the undiluted serum was mixed with solutions of the enzyme of 1 per cent and higher. None of these sera when injected into guinea pigs sensitized these animals passively to the enzyme. These experiments indicate that precipitins are not easily formed by these enzymes when they are injected into rabbits.

SUMMARY

It has been shown that trypsin from swine and cattle as well as chymotrypsin and chymotrypsinogen, crystallized by Northrop and Kunitz, will act as antigens in that they will actively sensitize guinea pigs. It has been further shown that these four substances can be differentiated, one from the other, by the anaphylactic test. In a limited number of experiments it was shown that precipitins are not readily formed when chymotrypsin and chymotrypsinogen are injected into rabbits.

BIBLIOGRAPHY

1. Kunitz, M., and Northrop, J. H., *Science*, **78**, 558 (1933).
2. Northrop, J. H., *J. Gen. Physiol.*, **13**, 739 (1929–30).
3. Sumner, J. B., and Kirk, J. S., *Science*, **74**, 102 (1931).
4. Kirk, J. S., *J. Biol. Chem.*, **100**, 667 (1933).
5. Kirk, J. S., and Sumner, J. B., *Proc. Soc. Exp. Biol. and Med.*, **29**, 712 (1931–32).
6. Dale, H. H., in Privy Council, Medical Research Council, A system of bacteriology in relation to medicine, London, **9**, 229 (1931).

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RELATION OF THE VIRUS AND THE INCLUSION BODIES OF SILKWORM "JAUNDICE"

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Throughout the progress of "jaundice" disease in silkworms large numbers of large inclusions (3–5 μ in dia.) termed polyhedral bodies or polyhedra are formed within the nuclei of certain tissue cells. These inclusions, their origin, and some of their properties have been described elsewhere (1). During the later stages of the disease the tissues disintegrate and liberated polyhedral bodies are found in the blood stream to the number of about a million and a half per 0.01 cc. These inclusions settle in water, so that by washing and centrifuging, several grams of them, in a partial state of purity, can be obtained from diseased cases. It was found that the polyhedra are nucleoprotein in nature (2), and that they do not harbor any constant organized elements in the morphological sense. Disease can be initiated in their absence (1, 3, 4), by separating them from the active principle by filtration or centrifugation. Because of this fact, the polyhedral bodies have been regarded by us as products of a specific disturbed metabolism; of diagnostic value, but of no etiological importance.

Except during the last stages of the disease, blood from jaundiced worms can be readily obtained under bacteriologically sterile conditions and it is a better source than excised tissues or emulsified cadavers. Extracts prepared from the latter must be sterilized by filtration and this at once introduces an uncertain factor. By the use of fresh diseased blood, however, a measurable quantity of infectious material may be removed and ampuled. Such blood contains the etiological agent of "jaundice," pathological blood cells, some cellular débris, and many polyhedral bodies, and it is highly infectious to normal silkworms. Our work (1) and that of Paillot (4) showed that when fresh blood from jaundiced cases is immediately diluted* and centrifuged or passed through Berkefeld candles to free it of polyhedral bodies, the resulting fluid is still infectious. Paillot further showed that blood from diseased worms is infectious long before the blood cells or plasma show evidence of the presence of polyhedra.

Notwithstanding the fact that a large amount of free virus exists in jaundiced worms, the suggestion presented itself that virus might also be adsorbed to or enclosed within the inclusions. Aoki and Chigasaki (5) shook and centrifuged the polyhedral bodies from jaundice in silkworms more than ten times in physiological salt solution and found that when so treated the inclusions were just as infectious on inoculation as the unwashed bodies. In 1927 we repeated this experiment and washed and centrifuged the polyhedra twenty-two times but failed to obtain jaundice in silkworms. This result has since been duplicated on a number of occasions and it is difficult to interpret. However, these preliminary experiments are not sufficient because the wash water was not tested and we had no measure of the activity of the virus which was presumably washed away. The present work, performed during three summer seasons, is an extension of the attempt to obtain the inclusions free from active jaundice virus.

General Methods

Virus blood ampuled and held at refrigerator temperature (0°–4°C.) for one year lost its infectivity only to a slight degree, and full activity was again restored after a single passage. The infectious material for these experiments was blood removed under sterile conditions from diseased silkworms after three or four serial passages of the virus through the worms.

For infections with blood or with washed and resuspended inclusion bodies 0.01 cc. of each dilution or suspension in water† was caught off the end of a 0.1 cc. serological pipette by means of a sterile and sharply pointed, standardized capillary tube. Each amount so caught was considered as an infective dose. The capillary tubes containing the 0.01 cc. amounts were sealed and put aside for a short interval until used. Washed or otherwise treated inclusion bodies were always handled in lots of 0.1 cc. of sediment and calibrated, prior to infection, in such wise that the dose of 0.01 cc. contained ± 100,000 inclusions. In order to accomplish this the number of bodies in samples of 0.01 cc. were always counted after the manner of making blood cell counts and adjusted to ± 100,000 per 0.01 cc. by proper dilution.

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* In work with small animals the blood must often be diluted to create a workable volume.

† Silkworms will not accept liquids containing detectable amounts of salt.

The silkworms used for experimentation were always of constant age (fourth stage) bred from an entirely healthy stock gradually produced during the past years. It is recognized that it is extremely difficult to rear large numbers of these insects disease-free, but by rigorous isolation and by using great precautions, the writers have succeeded in obtaining a thoroughly dependable stock of animals. When worms were needed, the desired number were segregated from the stock and each isolated in a sterile half-pint milk bottle covered with a paper cap. Food was withheld for 24 hours prior to infection. After this period each animal was restrained ventral side up and the infective dose held in the capillary tubes was gradually administered *per os*. By expelling minute amounts at a time from the pipettes and placing them upon the mouth parts, the fasted silkworms drank the entire 0.01 cc. doses in one or two minutes. When the heads were retracted in moulting, the silkworms could not drink, and a few resisted; these were discarded and replaced by more tractable individuals. After infection the worms again received their daily ration of mulberry leaves.

Uninfected silkworms in numbers equal to the infected animals were always held in the same room and in no case was any cross infection to the controls noted.

Removal of the Active Principle from the Blood by the Inclusions

Preliminary experiments showed that when infectious blood was permitted to stand vertically in the ampules the top layer lost its activity, whereas the bottom fraction containing the settled polyhedral bodies was highly infectious. Two more experiments were instituted to test these observations more rigorously.

Some diseased blood, which had remained in ampules undisturbed for 9 months, under refrigerator temperatures, was available. The clear upper fraction was separated from the settled polyhedra. Five silkworms were infected with 0.01 cc. of the polyhedra-free upper fraction and 5 worms received 0.01 cc. of this same fluid diluted 10 times with sterile water. The sedimented polyhedra were suspended in 0.1 cc. of sterile water and 10 worms were infected, each receiving 0.01 cc. containing ± 100,000 inclusions.

TABLE 1

Loss of Infectivity of Blood on Standing Vertically Nine Months

Upper fraction of blood					Sedimented bodies					
No.	Deaths from undiluted blood	Time in days infection to death	No.	Deaths from diluted blood	No.	Deaths	Time in days infection to death	No.	Deaths	Time in days infection to death
1	+	5	1	0	1	+	5	6	+	4
2	+	8	2	0	2	+	8	7	+	7
3	0		3	0	3	+	3	8	+	6
4	0		4	0	4	+	4	9	+	4
5	0		5	0	5	+	7	10	+	7

TABLE 2

Loss of Infectivity of Blood on Standing Vertically Five Days

Upper fraction of blood		Sedimented bodies		
No.	Deaths	No.	Deaths	Time in days infection to death
1	0	1	+	6
2	0	2	+	5
3	0	3	+	4
4	0	4	+	7
5	0	5	+	5
6	0	6	+	6
7	0	7	+	8
8	0	8	+	6
9	0	9	+	4
10	0	10	+	5

Table 1 gives the results of this experiment. Among the 10 worms which received the polyhedra-free fraction two contracted jaundice, whereas all of those which received the sedimented bodies died of the disease. Table 2 gives data for a similar experiment. In this case the infectious ampuled blood was permitted to stand at room temperature for 5 days. None of the silkworms which received 0.01 cc. of the upper, undiluted fraction of the blood contracted the disease, whereas the ten which received each 0.01 cc. of a water suspension containing ± 100,000 of the settled inclusions succumbed to jaundice.

These two experiments show that the virus of jaundice was almost completely removed from suspension in the blood by permitting the polyhedral bodies to settle slowly through this fluid. In time, this removal is probably accomplished by the adsorption of the virus to the surfaces of the inclusions. If infectious blood is drawn and the polyhedra immediately removed by centrifuging, the upper fraction retains its activity. This fact will be demonstrated as part of the last experiment, table 5, to be discussed later.

Attempt to Free the Inclusions of Virus by Prolonged Washing

Table 3 gives the data for two experiments performed with two separate lots of inclusion bodies.

One lot was used throughout the 23 tests in experiment 1; the other throughout the 23 tests in experiment 2. In both cases 0.1 cc. of polyhedral sediment, freed from blood, was initially used. This volume was gradually reduced during the progress of each experiment. This was unavoidable because some of the inclusions stuck to the glass tubes and were lost after each manipulation. Twenty-three tests were made for each of the 2 experiments and 5 silkworms were infected in each test. In table 3, test 1, 5 W.C. Super, signifies that the 0.1 cc. of polyhedra sediment was washed and centrifuged 5 times, each time in 5 cc. of separate volumes of sterile water and 0.01 cc. of the 5th supernatant fluid fed to 5 worms in each experiment. The washing involved suspension of the bodies in water by agitation of the sediment with a sterile pipette. A fresh pipette was used for each manipulation. The 5 cc. of wash waters 1 to 4 were discarded. In each experiment no cases of jaundice were obtained. In test 2, 5 W.C. + 5 H.S. + 1 C Super signifies that the same inclusions after discarding the supernatant fluid in test 1, were resuspended in 5 cc. of sterile water, and subjected to 5 hours of agitation in a shaking machine. 1 C Super means that the inclusions were then thrown down in the centrifuge tube and the supernatant fed, 0.01 cc. to each worm. One

TABLE 3

Code: C = centrifuging; W.C. = washing and centrifuging; H.S. = hours of shaking; Super = supernatant liquid or wash water; polyhedra = polyhedral inclusion bodies.

Test no.	Effect of repeated washings and shakings on the polyhedral bodies Infections with the washed bodies and with the wash water	No. worms used	Exp. 1 No. died	Exp. 2 No. died
1	5 W.C. Super	5	0	0
2	" + 5 H.S. + 1 C Super	"	0	1
3	" + " + 5 W.C. polyhedra	"	1	3
4	" + " + " + 5 H.S. + 1 C Super	"	4	0
5	" + " + " + " + 3 W.C. "	"	3	2
6	" + " + " + " + 5 W.C. "	"	2	1
7	" + " + " + " + " polyhedra	"	1	2
8	" + " + " + " + " + 5 H.S. + 1 C Super	"	0	0
9	" + " + " + " + " + " + 3 W.C. "	"	1	0
10	" + " + " + " + " + " + 5 W.C. "	"	2	1
11	" + " + " + " + " + " + " polyhedra	"	4	3
12	" + " + " + " + " + " + " + 5 H.S. + 1 C Super	"	0	1
13	" + " + " + " + " + " + " + " + 3 W.C. "	"	1	0
14	" + " + " + " + " + " + " + " + 5 W.C. "	"	0	0
15	" + " + " + " + " + " + " + " + " polyhedra	"	0	3
16	" + " + " + " + " + " + " + " + " + 5 H.S. + 1 C Super	"	0	4
17	" + " + " + " + " + " + " + " + " + " + 3 W.C. "	"	3	1
18	" + " + " + " + " + " + " + " + " + " + 5 W.C. "	"	2	2
19	" + " + " + " + " + " + " + " + " + " + " polyhedra	"	2	4
20	" + " + " + " + " + " + " + " + " + " + " + 5 H.S. + 1 C Super	"	2	4
21	" + " + " + " + " + " + " + " + " + " + " + " + 3 W.C. "	"	1	3
22	" + " + " + " + " + " + " + " + " + " + " + " + 5 W.C. "	"	1	0
23	" + " + " + " + " + " + " + " + " + " + " + " + " polyhedra	"	3	3

case of jaundice developed in experiment 2. In test 3, 5 W.C. + 5 H.S. + 5 W.C. polyhedra signifies that after the bodies were subjected to the washings, centrifugings, and shakings recorded in tests 1 and 2, they were washed and centrifuged 5 more times in 5 cc. of separate volumes of sterile water. In this test the first 4 wash waters were discarded and the polyhedra resuspended in the fifth by agitation with a pipette. These inclusions were then fed to 5 silkworms in each experiment in doses of 0.01 cc. containing ± 100,000 polyhedra. One case of jaundice developed in experiment 1 and 3 cases in experiment 2. In tests 4, 5, and 6 the treatment of the bodies was continued.

Five hours of shaking were added, the bodies were centrifuged down, and the 1st supernatant tested. More washings and centrifuging followed and the 3rd and 5th wash waters were tested, followed by infections with the polyhedra, in test 7, resuspended in 5 cc. of fresh sterile water.

The silkworms were all of uniform size and age. The wash water used always equalled 5 cc. and the dosage of wash water per worm was held at 0.01 cc. When infections were performed with the inclusions these were always fed in doses of 0.01 cc. water suspensions containing ± 100,000 bodies. The time of washing by agitating the polyhedra with a pipette consumed approximately 30 seconds. In centrifuging, the time and R.P.M. were also held constant at 5 minutes and 1500 respectively.

Samples of the polyhedral bodies were examined microscopically at intervals during the prolonged course of treatment and even after the two 23rd tests showed no visible changes such as cracking or fragmentation. This was not surprising in view of the well known fact that the polyhedral bodies are quite resistant to dyes, moderately high pressures, and solution in water. These facts are at variance with what is known of the apparently delicate natures of the intracellular inclusions found in the virus diseases of many of the higher animals.

The experiment outlined showed that the washings and shakings freed considerable amounts of virus from the surfaces of the polyhedral bodies, but that it was impossible to remove it entirely. In general, the polyhedra were just as infectious after the 23rd treatment as after the preceding treatments. Since worms fed 0.01 cc. of undiluted and untreated blood yield approximately 100 per cent mortality, the prolonged treatment to which the bodies were subjected did not entirely remove the adsorbed virus, although virus was liberated, as the results with the wash waters showed.

The cleansing experiment was repeated but the pH of the wash waters was varied from 5.0–6.8. Surface changes or agglutinations of the bodies occurred when the medium was too acid or too alkaline, but at the range employed no visible effect on the inclusions was noticed. It was also known that the H-ion concentrations used did not affect the activity of the virus. The final outcome of this experiment corresponded to the previous one; virus was liberated into the wash waters, but the polyhedra remained infectious.

TABLE 4

Titration Experiments

Amount of virus blood administered*	Exp. 1		Exp. 2		Exp. 3		Exp. 4		Exp. 5		Exp. 6		Total no. fed	Total no. died	Per cent mortality
	No. fed	No. died	No. fed	No. died	No. fed	No. died	No. fed	No. died	No. fed	No. died	No. fed	No. died			
10^{-12}	2	0	2	0	3	0	4	0	5	0	5	0	21	0	0
10^{-11}	2	0	2	0	3	1	4	1	5	1	5	0	21	3	14
10^{-10}	2	1	2	0	3	0	4	0	5	0	5	1	21	2	10
10^{-9}	2	0	2	1	3	0	4	0	5	1	5	0	21	2	10
10^{-8}	2	1	2	0	3	0	4	1	5	2	5	0	21	4	19
10^{-7}	2	0	2	0	3	0	4	1	5	0	5	1	21	2	10
10^{-6}	2	0	2	0	3	2	4	0	5	1	5	1	21	4	19
10^{-5}	2	1	2	2	3	0	4	1	5	2	5	1	21	7	33
10^{-4}	2	1	2	1	3	2	4	3	5	2	5	1	21	10	48
10^{-3}	2	2	2	2	3	3	4	3	5	4	5	2	21	16	76
Total	20	6	20	6	30	8	40	10	50	13	50	7	210	50	

* Controls fed corresponding amounts of normal blood were held; 6 silkworms for each dilution. None of the 60 silkworms so treated died. As untreated controls, to determine the possibility of cross infection, 150 silkworms were held in the room during the experiment. None of these died.

The results given, especially at the end of the above experiments, could not have been due to high dilutions of some of the original infectious blood. The small amounts of blood adhering to the polyhedra must have been washed away very soon because each wash water was discarded completely after the infection was performed and fresh, sterile water added prior to the next manipulation. Therefore, the argument that a titration of the original trace of blood was made cannot be used. The wash waters which produced jaundice must have received the virus from the inclusions or in cases in which the inclusions themselves were used the virus must have been associated with them. The number of cases of disease obtained at the end of the experiments did not differ materially from the number obtained at the beginning. It was thought well, however, to determine how far infectious blood can be diluted and still remain active. Table 4 gives the results of 6 separate experiments performed by progressively diluting infectious blood with sterile water. It was found that this blood will occasionally titrate to a dilution of 1 part of virus blood in one hundred billion parts of water. At 1 to a trillion dilution the activity of the infectious agent was definitely lost. In other words, virus blood can be greatly diluted, but not to the extent which would account for the cases of jaundice obtained at the end of the washing experiments. The actual dilution finally obtained with the 35 washings cannot be calculated, because the wash waters were always discarded and were not progressively diluted.

Attempt to Free the Inclusions of Virus by Chemical Agents

The possibility of freeing the polyhedra of virus by means of chemical agents was next considered. Some inclusions were shaken with water suspensions of willow carbon. Infection experiments showed that the carbon adsorbed virus, but we were unable to free the polyhedra of the infectious agent by this method. Alcohol (80 and 95 per cent), iodine solution and 5 per cent potassium dichromate were tried over various periods of time, but without effect. Five per cent phenol used for 1 hour did not sterilize the polyhedra. If the infectious material had been in a thoroughly dry state prior to treating with alcohol and other agents the results might have been different, but drying was thought inadvisable for the purposes of the present experiments.

Inactivation of Polyhedra by Heat and Readsorption of Free Virus to the Bodies

Polyhedral bodies were washed and centrifuged 5 times and then suspended in water, the tubes sealed and submerged in heated water. Two experiments showed that the inclusions were not always sterilized when they were heated for ½ to 1 hour at 60°C. When maintained at 100°C. for ½ hour, however, the polyhedra were rendered inactive. Table 5 shows 3 separate tests involving 5 worms for each test with 3 separate lots of polyhedra sterilized by heat, involving the infection of 15 worms.

Each silkworm in this group received ± 100,000 inclusions, but no jaundice developed. Fresh infectious blood was then freed of its natural polyhedra by quickly centrifuging. This freed blood proved highly infectious, as shown by the deaths from jaundice of 14 of the 15 worms infected during the course of 3 trials of 5 worms each.

TABLE 5

Effect of Passing Polyhedra Sterilized by Heat through Active Virus Blood

	Number of worms fed	Number died	Per cent mortality
Heated polyhedra	15	0	0
Virus blood freed of polyhedra by centrifuging	15	14	93
Virus blood after sedimentation of heated polyhedra	15	4	27
Remaining heated polyhedra centrifuged from virus blood after sedimentation (not washed)	15	8	53
Supernatant I	15	4	27
Supernatant III	15	0	0
Supernatant V	15	0	0
Heated polyhedra sedimented through virus blood and washed five times	15	5	33

The sterilized polyhedra were then permitted to settle, for 24 hours, through this active, polyhedra-free blood and in so doing a considerable amount of virus was removed. This is shown in the 3 tests with 0.01 cc. of the clear, upper liquid which only yielded 4 cases of jaundice. It was found, however, that many of the bodies still remained in suspension after the 24 hours sedimentation, so the remaining ones were removed by throwing them down in a centrifuge tube. These bodies were not washed and were found active. The main bulk of the heated polyhedra which had settled out during the 24 hours was washed and centrifuged 5 times in 5 cc. of separate volumes of sterile water. The supernatant liquids or wash waters 1, 3, and 5 were tested on silkworms by feeding 0.01 cc. amounts, but the first was the only infectious one. The washed polyhedral bodies were then tested by feeding 0.01 cc. water suspensions containing ± 100,000 and 5 cases of jaundice developed.

The above experiment demonstrated that the virus associated with the polyhedra was destroyed by heat and that some was adsorbed to the treated bodies when they were brought in contact with active virus. That more virus was not adsorbed may have been due to changes in the surfaces of the bodies produced by the heat.

We have been unable to demonstrate the presence or absence of active virus within the polyhedral bodies, and, at present, know of no technique which would satisfactorily test this possibility. All of the results obtained can be explained, we believe, on the basis of surface adsorption of the virus to the protein material of the inclusions. Therefore, we see no reason for changing our previous point of view, namely, that the polyhedral bodies are reaction or by-products of the disease. It is true, they have an affinity for the virus, but that does not mean that they are genetically related to it or that they can be compared to a cyst-like secretion by an active agent within. It has been repeatedly shown, in this as well as in previous filtration work, that the disease can be produced without the polyhedra, so no need exists for attributing to them any direct etiological relationship to it.

SUMMARY

When freshly drawn jaundice blood, containing polyhedral bodies, was immediately centrifuged, the supernatant fluid freed from the inclusions was highly active. If infectious blood was permitted to stand for some time the inclusion bodies settled and in the upper layer of the fluid which was free from them, no virus was found. The polyhedra continuously lost virus when washed in water, but could not be freed from the infective agent in this manner. A similar result was obtained when the reaction of the wash water was purposely varied. The results with the wash waters could not have been due to a high dilution of traces of the original infectious blood. Though diseased blood was infectious occasionally when diluted 10^{-11} times it lost its activity at 10^{-12}.

The polyhedra could not be freed of virus by the use of certain chemical agents. Heat, however, was effective in rendering inactive

the virus associated with them. When they were now brought in contact with active polyhedra-free blood, some of the virus became associated with them.

The inclusions are evidently physical carriers of the virus, but the experiments do not lend support to the view that they are etiologically important in the sense of a genetic relationship.

BIBLIOGRAPHY

1. Glaser, R. W.
1915. Jour. Agric. Res., *4*, 101.
Glaser, R. W.
1927. Ann. Ent. Soc. Am., *20*, 315.
Glaser, R. W.
1928. Virus Diseases of Insects, in "Filterable Viruses," edited by Thomas Rivers, Williams & Wilkins Co., Baltimore, Md.
Glaser, R. W., and Cowdry, E. V.
1928. Jour. Exp. Med., *47*, 829.
2. Glaser, R. W., and Chapman, J. W.
1916. Biol. Bull., *30*, 367.
3. Acqua, C.
1918–19. Rend. Inst. bact. R. Scuola Super. Agric. Portici, *3*, 243.
4. Paillot, A.
1924. Comp. rend. Acad. Sci., *179*, 229.
Paillot, A.
1926. Ann. Inst. Pasteur, *40*, 314.
5. Aoki, K., and Chigasaki, Y.
1921. Centrlbl. Bakt. u. Parasitenk., Abt. I, Orig., *86*, 481.

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THE DECOMPOSITION OF CITRIC ACID BY BACILLUS AERTRYCKE

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The ease with which certain pathogenic bacteria grow in media composed of simple chemical substances, such as ammonium citrate and a few inorganic salts, presents the possibility of a detailed study of the chemical changes caused by these bacteria. Since many non-pathogenic organisms also are known to decompose such media, a comparison of their action with that of the pathogens can be made. From relatively simple salts, the bacteria are able to synthesize all the complex materials which are necessary for their growth. As a result of bacterial action, the substrate is decomposed into smaller molecules, some of which can be isolated and identified.

Some results of a study of the decomposition of citric acid by a laboratory strain of *Bacillus aertrycke* are reported here. This bacillus is a member of the paratyphoid Type B group, pathogens which have the special ability to grow well on a synthetic citrate medium (1). This medium is not readily contaminated, and is composed only of well known chemically pure substances.

The course of the decomposition of citric acid by *Bacillus aertrycke* was followed by the use of a convenient procedure for the determination of citrate which is described elsewhere (2). Examination of the medium at various stages of the decomposition showed that formic and acetic acids were produced early in the process. 1 mole of citric acid through the action of the bacteria gave rise to 0.25 mole of formic acid, 1.5 moles of acetic acid, and nearly 0.75 mole of succinic acid, in addition to carbon dioxide. Study of the volatile acid fraction showed that under the conditions used, formic and acetic acids were the only volatile acids produced in appreciable amounts. This fraction was isolated by distillation *in vacuo*, a method which, with certain precautions, has proved both rapid and reasonably accurate. Succinic acid was then isolated from the residue by extraction with ether.

The results of this study differ from those reported in the literature concerning the action of other organisms on citric acid, first, in the demonstrated presence of formic acid; second, in the rapid production of relatively large amounts of succinic acid; third, in the somewhat diminished production of acetic acid; fourth, in the absence of demonstrable amounts of acetonedicarboxylic acid or acetone resulting from its decomposition; and fifth, in a comparison of the products formed by the action of rough and smooth forms of the same organism, with the identification of lactic acid produced by the smooth form.

Studies of the products of decomposition of citric acid by other organisms have been made by a number of workers. Terada (3) obtained a rather large yield of succinic acid by the prolonged action of an unclassified organism from the air. The action of *Bacillus suipestifer* on citrate in the presence of peptone has been studied by Brown, Duncan, and Henry (4) who identified acetic acid, carbon dioxide, and a trace of succinic acid as the products. Some of the volatile acid was formed from the peptone. Bosworth and Prucha (5) reported that the decomposition of citric acid in milk by *Bacillus lactis aerogenes* yields 2 moles of acetic acid for 1 of citric acid. Butterworth and Walker (6) in studying the action of *Bacillus pyocyaneus* on citric acid found acetonedicarboxylic acid and acetone produced by its partial decomposition; malonic and acetic acids were then isolated and a trace of succinic acid was found.

In the decomposition of citrate by *Aspergillus niger*, Walker, Subramaniam, and Challenger (7) have established a series of products which likewise begins with acetonedicarboxylic acid. This is followed in turn by malonic, acetic, glycolic, glyoxylic, and finally oxalic acid. No succinic acid was reported. Wieland and Sonderhoff (8) studied the anaerobic oxidation of citric acid by yeast. They found acetic acid and a small amount of succinic acid, but were unable to show that hydrogen was a product of the decomposition, although they had reason to expect it.

EXPERIMENTAL

Synthetic Bacteriological Culture Medium

A medium similar to those suggested by Braun and Cahn-Bronner (9), Pesch (10), and Koser (1) has been prepared. Excellent growth was secured with several strains of *Bacillus paratyphosus* B and *Bacillus aertrycke* in the medium, prepared from chemically pure salts, of the following composition.

Diammonium citrate, anhydrous, *gm.*	1.0
Dipotassium phosphate, " "	1.0
Sodium chloride, *gm.*	0.5
Magnesium sulfate, hydrated, *gm.*	0.1
Ferric chloride 0.01 per cent, *drops*	3
Calcium " 0.01 " " "	3
Distilled water, *ml.*	100
pH	6.0

The medium was filtered and sterilized by autoclaving at 120° for half an hour. For the purposes of this study, it proved desirable to subculture in the synthetic medium at least ten times in order to secure rapid and uniform growth from small inocula. Tubes of 50 ml. capacity containing 15 ml. of medium were sterilized and used for transfers, which were made with a 2 mm. platinum loop. Incubation at 37° of a 100 ml. portion inoculated in the same way resulted in a heavy turbidity in 24 hours with disappearance of nearly all of the citrate. The medium became more alkaline. Growth was particularly heavy at the surface and took place best when the medium was exposed to the air in shallow layers.

A bacteriological study of the stock culture after 233 transfers in the synthetic medium was secured through the kindness of Dr. Carl Ten Broeck. The organisms consisted of rough and smooth forms of *Bacillus aertrycke.* The former, which greatly predominated, were motile and in part greatly elongated. The latter were short motile rods, more actively motile when transferred to broth. Both forms produced gas from glucose, the rough form acting much more rapidly. Both forms were agglutinated with specific immune serum in dilutions of 1:12,000 for the smooth strain and 1:50,000 for the rough strain, in which there was a tendency to spontaneous agglutination; this dilution was near the limit for the immune serum which was used.

Methods and Apparatus—For chemical study 1 liter of solution in a 3 liter round bottomed sterile flask plugged loosely with cotton was inoculated with the platinum loop and incubated at 37° or in some cases at 34°. At intervals 100 ml. portions were withdrawn under sterile conditions for chemical examination. Determination of residual citrate, together with formic, acetic, and succinic acids, was made.

Duplicate determination of citrate in the sample required 2 ml. and was accomplished by the use of Denigès' reaction (2). The remaining 98 ml. were filtered through a Seitz filter with two 5 ml. washings, giving a water-clear filtrate. Volatile acidity was determined by adding 9 ml. of 20 per cent phosphoric acid and distilling in a modified Claisen flask at a water pump vacuum of about 20 mm. The distilling flask had a capacity of 250 ml. and was provided with a 20 cm. indented tubulation. When the residual material had largely solidified, two 15 ml. portions of water were added and distilled separately to sweep over any residual volatile acid. The volume of the distillate was determined and 25 ml. taken in duplicate for titration with standard sodium hydroxide. The end-point was determined in boiled solution with phenolphthalein as indicator. Phosphate was uniformly absent, but variable amounts of hydrochloric acid, produced from the sodium chloride of the medium, were present. To the neutralized sample was therefore added 1 ml. of 10 per cent potassium chromate, chloride ion was titrated with 0.05 N silver nitrate, and the total acidity was corrected for the amount of hydrochloric acid thus determined.

Formic acid was determined in 2 ml. of the distillate by adding 2 ml. of the mercuric chloride-sodium acetate reagent of Franzen and Egger (11). The solution heated at 100° for 4 hours in a centrifuge tube deposited calomel, which was washed, dried, and weighed in the same vessel.

The non-volatile residue from the distillation was transferred to a long 30 ml. test-tube by rinsing with three or four 5 ml. portions of hot water. Two short, dense plugs of absorbent cotton between which a layer of anhydrous sodium sulfate was inserted were next placed at the mouth of the tube, well above the fluid, and around the funnel tube used in ether extraction (12). The whole was then continuously extracted with ether for 24 to 36 hours. The receiver was changed at the end of 5 hours in order to obtain some fractionation of the extract.

The procedure was the outcome of study of many cultures. It was found necessary to remove the bacteria by filtration in order (*a*) to avoid excessive foaming which sometimes occurred during distillation in the presence of bacterial bodies, (*b*) to lessen the production of emulsions during ether extraction, and (*c*) to avoid the introduction of impurities extracted from the bacterial bodies. Since filtration was frequently slow, the effects of using the unfiltered culture were studied. There was no significant change either in the quality or quantity of the products. This was determined by dividing a 200 ml. culture into 2 equal parts, one of which was filtered, the other not. The amounts of acetic and formic acids from each were the same. The non-volatile residue from the filtered specimen gave a nearly clear water solution, but that from the unfiltered part upon addition of water left a wax-like material which weighed less than 0.01 gm., and was readily separated by filtration. The foaming which occasionally became serious during the distillation of this sample was controlled by the addition of a drop of octyl alcohol as often as necessary. Control tests showed that this did not alter the amount of either fraction.

The formation of emulsions during ether extraction caused little difficulty with the extracting arrangement described. In spite of the absence of mechanical entrainment of phosphoric acid, after long extraction a detectable amount of phosphate was found in the residue. Since, when the decomposition of citrate is complete, the non-volatile fraction is all extracted in 5 hours, this difficulty has not in general been found serious.

In order to show the reliability of the method of volatile acid determination, studies were made of the recovery of known mixtures of the acids identified below when distilled with phosphoric acid. By the procedure described, 85 per cent of the volatile acid was recovered in one distillation, and with two washings at least 96 per cent was secured. The missing portion was largely acetic acid.

Identification and determination of the products were made as follows: The presence of formic acid in the distillate was shown not only by reduction of mercuric chloride to calomel in the reaction used for its determination, but also by the reduction of methylene blue in the presence of sodium sulfite (13). Acetic acid was identified by the preparation of *p*-nitrobenzyl acetate, melting at 76–79°, from the sodium salt of the distillate and *p*-nitrobenzyl bromide. A mixed melting point with an authentic sample (78–80°) showed no depression. By subtracting from the standard alkali consumed by the distillate the part due to hydrochloric and formic acids, the amount used by the remaining volatile acid was obtained. This was then calculated as acetic acid.

The conclusions concerning the nature and amount of these volatile acids were confirmed by a Duclaux analysis carried out by the method of Virtanen and Pulkki (14). After 33 hours, a 100 ml. sample of culture was removed from 1 liter of medium inoculated with Subculture 153. The volatile acid distillate, including washings, was 150 ml. Of this, 50 ml. were used for determining titratable acidity, chloride, and formate. This analysis showed that the volatile acids present in 100 ml. were equivalent to 2.2 ml. of 0.1 N HCl, 22.6 ml. of 0.1 N formic acid, and 86.0 ml. of 0.1 N acetic acid. By using the known semidistillation constants for the organic acids and neglecting the hydrochloric acid, the amount of 0.1 N NaOH necessary to neutralize this distillate was calculated to be 36.7 for 50 ml. The observed value was 37.2 ml.

While less than 0.05 ml. of 0.05 N hydrochloric acid appeared in the distillate during semidistillation, the effect of this acid on the constants of the organic acids was unknown. Decreasing ionization would no doubt slightly increase the semidistillation constants. The constants for the particular apparatus used were not determined. The agreement between calculated and observed values is therefore considered to indicate, within the experimental error, that no other volatile acid was present in significant amounts.

The non-volatile residue from cultures grown under the conditions already described consisted of succinic acid in amounts varying for the most part between 0.1 and 0.3 per cent. The succinic acid obtained on evaporation of the ether extracts was remarkably pure, showing a melting point of 180–182° without further treatment. One crystallization from water sufficed to give an analytically pure sample melting at 187.5° (corrected). A titration equivalent of 59.8 was found (theory 59.0). The methyl ester, prepared by the action of a slight excess of diazomethane, melted at 20° and gave no depression with a known sample.

The quantitative relation between the decomposition products of citric acid at various stages of the growth of the culture is shown in Table I.

In many experiments, the amount of acetic acid was slightly higher than in this case, and the amount of succinic acid between 1 and 2 millimoles, with not much variation of the quantity of formic acid. Carbon dioxide was present whenever tests for it were made. It was identified by the formation of barium carbonate from the CO_2-free air used in aerating some of the cultures.

The distribution of carbon among the various decomposition products of citric acid was studied with both the rough and the pure smooth strains of *Bacillus aertrycke* isolated from the laboratory strain previously described, after 233 transfers on synthetic citrate medium. Table II shows the carbon distribution found in 100 ml. cultures incubated at 37° after inoculation from a suspension of these forms. The carbon dioxide was absorbed in standard barium hydroxide by aerating, acidifying, and further aerating with carbon dioxide-free air until no more barium carbonate was precipitated. The amount of bacterial bodies was estimated by filtering 10 ml. of the acidified culture through a weighed platinum Munroe crucible, which removed most of the turbidity. After washing thoroughly and drying at 100° *in vacuo* for 5 hours, the difference in weight multiplied by 10 was taken to be that of the bacterial bodies, for which a carbon content of 50 per cent was assumed.

TABLE I

Products Formed during Growth of Bacillus aertrycke on Citric Acid

Incubation time	Citric acid	Acetic acid	Formic acid	Succinic acid
hrs.	*mM*	*mM*	*mM*	*mM*
0	4.43	0	0	0
19	3.32	1.6	0.35	
33	0	6.95	1.02	3.06

The carbon balance for the rough form of the organism shows the distribution of 95 per cent of the carbon initially introduced. In addition, 1 per cent was used for the determination of citric acid; 96 per cent is thus accounted for. For the smooth form 92 per cent of the initial carbon is accounted for in the products, and an additional 1 per cent was used for the citric acid estimation. The chief sources of error appear to lie in the determination of the weight of the bacterial bodies and perhaps in the incomplete extraction of the non-volatile acid fraction. Lactic acid was identified regularly in smooth cultures, but not in rough cultures or in the ordinary *Bacillus aertrycke* culture under the conditions used.

TABLE II

Carbon Distribution in Synthetic Citrate Medium after Incubation with Rough and Smooth Forms of Bacillus aertrycke

Substance found	Rough form (1 loop inoculum)				Smooth form (0.5 ml. inoculum)			
	Amount after		Carbon after		Amount after		Carbon after	
	0 hrs.	40 hrs.	0 hrs.	40 hrs.	0 hrs.	17 hrs.	0 hrs.	17 hrs.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Acids								
Carbonic (CO_2)...	0	376	0	102.4	0	252	0	68.6
Formic...........	0	23	0	6.0	0	75	0	19.4
Acetic............	0	199	0	79.5	0	372	0	148.0
Succinic..........	0	130	0	53.0	0	65	0	26.5
Lactic............	0	0	0	0	0	35	0	14.3
Citric............	850	110	319	42.0	850	0	319	0
Bacteria............	0	40	0	20.0	0	30	0	15.0
Total............			319	302.9			319	291.8

Identification was made by the thiophene reaction and was substantiated by the guaiacol, codeine, and acetaldehyde tests (15).

A comparison of the rough and smooth strains through a number of experiments showed that the rough type regularly had a denser, more rapid growth; produced more succinic acid; formed less formic acid, in some cases none whatever; formed less acetic acid; evolved more carbon dioxide; and produced no lactic acid. A further comparison of these forms is being made.

DISCUSSION

The identification of formic, acetic, lactic, succinic, and carbonic acids in a synthetic citrate medium inoculated with *Bacillus aer-*

trycke shows that a very thorough decomposition of the citrate molecule has taken place. The precise manner in which these substances are formed is not yet clear, but important processes are evidently concerned with oxidation, since the most rapid action occurs in the aerated culture. Since acetonedicarboxylic acid can be obtained from citric acid by the action of various oxidizing reagents, this acid might be a product of the bacterial action. No evidence of its presence was found, however, nor of acetone, into which it readily decomposes. Moreover, growth on a medium composed of acetonedicarboxylic acid and the requisite salts both with and without formate was feeble, and produced no non-volatile and little volatile acid. It therefore appears improbable that this substance is an intermediate in the decomposition. In view of the identification of lactic acid, the possibility that pyruvic acid or aldehyde may be an intermediate (16, 17) deserves investigation.

The formation of succinic acid in relatively large amounts appears to require some other mechanism than that proposed by Brown, Duncan, and Henry (4), who assumed free radicals as intermediates. Grey (18) had proposed the same mechanism to explain the action of *Bacillus coli* on citrate in the presence of calcium formate, by which acetic and succinic acids were formed. Butterworth and Walker (6) have likewise resorted to the assumption of free radical formation. It is evident from the equations proposed by Brown, Duncan, and Henry that for every molecule of succinic acid isolated, a molecule of formic acid should be found. The data in Table I show, however, that this is not the case, for 3 times as many molecules of succinic acid as of formic acid were isolated. The separate rough and smooth forms likewise fail to produce the expected ratio (Table II). The bacteria are able to utilize succinate but not formate under these experimental conditions, so that the amount of formic acid remains nearly constant in a series of experiments, while the quantity of succinic acid varies. The mechanism proposed by these authors therefore does not appear to explain the decomposition of citric acid by *Bacillus aertrycke.*

The formation of succinic acid from the protein constituents of the bacteria (19) does not account for the data under discussion, since the amount of acid is far greater than the total amount of protein. Acetic acid can scarcely be the stage preceding succinic acid (16, 20, 21) for in synthetic media the bacteria fail to utilize acetate at hydrogen ion concentrations more acid than pH 7, whereas the optimum pH for the production of succinate lies between 5 and 6. Whether a hydroxyadipic acid (17) may be the precursor in this case, there are not sufficient data to decide. A substituted acetic acid is also worth consideration (22) in a discussion of this question.

While both the rough and smooth forms produce succinic acid, the former gives a larger yield and acts more rapidly; the inoculum of the smooth form was much larger than that of the rough in the experiments shown in Table II. The rough form produces much less formic acid than the smooth, and appreciably less acetic acid. The cultural differences between these forms are those commonly found between rough and smooth forms; the rough is more resistant, and the smooth is more sensitive to changes in environment. Both forms have maintained their cultural and biochemical characteristics in this simple medium through twenty transfers. The more selective action of the smooth form is no doubt responsible for the isolation of lactic acid. Since the substance isolated is optically inactive, it may be the result of a dismutation or other non-enzymatic reaction which has time to occur in the smooth but not the rough culture.

SUMMARY

1. A study was made of the chemical changes which occur when *Bacillus aertrycke* is cultivated on a synthetic citrate medium with ample exposure to the air.

2. The products identified were formic, acetic, and succinic acids and carbon dioxide.

3. The ratio of succinic to formic acid which was found renders improbable the assumption that free radicals play an important part in the decomposition of citrate by bacteria.

4. Rapid and convenient methods for the determination of citric and acetic acids were developed.

5. Comparison of the rough and smooth forms of *Bacillus aertrycke* showed that the smooth form produces more formic and acetic acids, less carbon dioxide and succinic acid; and in addition, small amounts of lactic acid.

BIBLIOGRAPHY

1. Koser, S. A., *J. Bact.*, **8**, 493 (1923).
2. Bruce, W. F., *Ind. and Eng. Chem.*, *Anal. Ed.*, **6**, 283 (1934).
3. Terada, S., *J. Pharm. Soc. Japan*, **511**, 697 (1924).
4. Brown, H. C., Duncan, J. T., and Henry, T. A., *J. Hyg.*, **23**, 6 (1924).
5. Bosworth, A. W., and Prucha, M. J., *J. Biol. Chem.*, **8**, 479 (1910).
6. Butterworth, J., and Walker, T. K., *Biochem. J.*, **23**, 926 (1929).
7. Walker, T. K., Subramaniam, V., and Challenger, F., *J. Chem. Soc.*, 200, 3044 (1927).
8. Wieland, H., and Sonderhoff, R., *Ann. Chem.*, **503**, 61 (1933).
9. Braun, H., and Cahn-Bronner, C. E., *Centr. Bakt.*, *1. Abt.*, *Orig.*, **86**, 196 (1921); *Klin. Woch.*, **1**, 1824 (1922); *Biochem. Z.*, **121**, 309 (1922).
10. Pesch, K., *Centr. Bakt.*, *1. Abt.*, *Orig.*, **86**, 97 (1921).
11. Franzen, H., and Egger, F., *J. prakt. Chem.*, **83**, 323 (1911).
12. McNair, J. B., *Ind. and Fng. Chem.*, *Anal. Ed.*, **5**, 62 (1933). Quick, A. J., *Ind. and Eng. Chem.*, *Anal. Ed.*, **5**, 76 (1933).
13. Denigès, G., *Bull. Soc. chim.*, **51**, 685 (1912).
14. Virtanen, A. I., and Pulkki, L., *J. Am. Chem. Soc.*, **50**, 3138 (1928).
15. Bernhauer, K., Die oxydativen Gärungen, Berlin, 165 (1932).
16. de Graaf, W. C., and Le Fevre, A. J., *Biochem. Z.*, **155**, 313 (1925).
17. Bernhauer, K., and Iglauer, A., *Biochem. Z.*, **249**, 211 (1932).
18. Grey, E. C., *Proc. Roy. Soc. London, Series B*, **96**, 156 (1924).
19. Ehrlich, F., *Biochem. Z.*, **18**, 391 (1909).
20. Moritz, C., and Wolffenstein, R., *Ber. chem. Ges.*, **32**, 2534 (1899).
21. Bernhauer, K., and Stein, W., *Biochem. Z.*, **249**, 219 (1932).
22. Panizzon, L., *Helv. chim. acta*, **15**, 1187 (1932). Fichter, E., and Panizzon, L., *Helv. chim. acta*, **15**, 996 (1932).

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THE INFLUENCE OF INHERITANCE AND ENVIRONMENT ON THE MILK PRODUCTION AND BUTTERFAT PERCENTAGE OF JERSEY CATTLE

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INTRODUCTION

In the Island of Jersey the importation of cattle has been prohibited since 1763, or for about 40 generations in the life scale of cattle. The Royal Jersey Agricultural Society has systematically tried to standardize the animals by drawing up scales of points and guiding the breeding operations of individual farmers. The Jerseys of North America were imported from this island stock, mostly during the last quarter of the nineteenth century and the present century. The million or so progeny of these imported cattle have been kept separate from other cattle by registration of pure breeding.

From 1900 to 1923 about 14,000 of the half million American Jersey cows[1] were tested for their milk yield over a year's lactation. All of these cows were able to produce the minimum requirement of 360 pounds of butterfat at 5 years of age. Their performance records include the cow's name and number, the name and number of her sire and dam, her age, the length of her record, the amount of milk and butterfat that she produced, the butterfat percentage, and the duration of time she carried her calf. The purpose of the present investigation was so to analyze these records of milk secretion as to ascertain the part played by the inheritance of each ancestor and by the environment in fixing the yield and butterfat percentage of a cow's milk.

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[1] Registry of Merit of Jersey Cattle, American Jersey Cattle Club, New York.

The records chosen for study were those of 364, 365, or 366 days. All records were corrected for age.[2] Beyond a correction to the equivalent expected record of 8 years and 3 months, however, no changes in the data were made. While this group represents but a small proportion of the total number, in fact a proportion hardly adequate for a concerted genetic control of the breeding for milk production within this breed as a whole, it does not appear to be heavily selected. The data are, therefore, treated as though selection were absent. The problem with which this paper deals may be illustrated by the pedigrees described below.

Pedigrees of High and Low Milk Production

The sum total of the effects of inheritance or environment on milk secretion may be seen in a typical pedigree of a high milk-producing cow as contrasted with one of low milk production. To make such pedigrees, all the Jersey cows whose mature-form production was under 8,000 pounds were taken as the low-producing group. There were 766 such cows in the 3,753 animals which came from tested dams. For the high-producing group all cows were taken whose production was over 12,000 pounds. There were 738 such cows in the 3,753 animals which came from tested dams. The pedigrees of each cow were run out to the third generation. The average mature-form production of the daughters of each animal in the pedigree was then determined, summed, and averaged. This average appears in the pedigrees presented below. Besides this average, the average production of the dams, the paternal granddams, and the maternal granddams was found and that average also appears in the pedigree.

A glance at the pedigree of the average low-producing cow as compared with that of the average high-producing cow shows that throughout the production of the ancestors of the high-producing animals was distinctly greater than that of the low-producing animals. It will be further noticed that on the whole the records of the parents are more nearly like those of their progeny than like those of the grandparents. There is, unfortunately, a selective factor which to some degree impairs this comparison. The cows which were selected for this group of necessity appear in the averages for the daughters of the sire or dam of that group. Similarly, the high-producing cows

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[2] While the actual correction constants were determined specifically for these Jersey cattle data from their own mean age production curves, they are in principle the same as those developed by the writer elsewhere (12).[3]

[3] Reference is made by number (italic) to Literature Cited, p. 554.

Low Milk Yield

- Daughters' milk yields under 8,000 pounds.
  - Sire; daughters' average milk yield 9,376 pounds.
    - Paternal grandsire; daughters' average milk yield 9,844 pounds.
    - Paternal granddam; daughters' average milk yield 10,063 pounds, average paternal granddam's milk yield 10,327 pounds.
  - Dam; daughters' average milk yield 8,639 pounds, average dam's milk yield 8,945 pounds.
    - Maternal grandsire; daughters' average milk yield 9,401 pounds.
    - Maternal granddam; daughters' average milk yield 9,299 pounds, average maternal granddam's milk yield 9,129 pounds.

High Milk Yield

- Daughters' milk yields above 12,000 pounds.
  - Sire; daughters' average milk yield 10,572 pounds.
    - Paternal grandsire; daughters' average milk yield 10,265 pounds.
    - Paternal granddam; daughters' average milk yield 11,183 pounds, average paternal granddam's milk yield 12,044 pounds.
  - Dam; daughters' average milk yield 11,858 pounds, average dam's milk yield 10,890 pounds.
    - Maternal grandsire; daughters' average milk yield 10,212 pounds.
    - Maternal granddam; daughters' average milk yield 10,710 pounds, average maternal granddam's milk yield 10,062 pounds.

appear in the averages as daughters of their parents. The effect on the average record of the sires' daughters is not pronounced since these selected cows are only one-fifth to one-seventh of the whole number of daughters. The effect on the average record for the dams' daughters is quite marked since these selected low- or high-producing daughters are a half of the whole number from which the average is obtained. There appears to be no really satisfactory way to overcome this difficulty. The reader should keep it clearly in mind, however, and realize that the apparent difference in the production of the sire's daughters and dam's daughters has no biological basis but is due only to the particular arrangement of the data. Fortunately this difficulty does not affect the records of the grandparents or the average production of the dams' own records. The marked selection of sires on the basis of the record of their dams is apparent in both pedigrees. The average production of the daughters of all the grandparents is higher for the high-group granddaughters than for the low-group granddaughters. For each given group, the daughters of the paternal and maternal grandsires are about equal in production.

An examination of the actual production of the females of the pedigree shows that the daughters' production resembles that of their dams rather more than that of the grandparents. The prominent selection of the paternal granddams is evident. The maternal granddams are relatively little selected. Their milk yields show a definite similarity to those of the granddaughter, although this similarity is not so great as that of the dams.

Similar pedigrees showing the sum total effect of inheritance and environment on the butterfat percentage of the daughter are presented below. To form the low-testing pedigree 415 Jersey cows were taken that had a butterfat test under 4.8 percent. These cows came from tested dams and were by sires which had tested daughters. This group constituted about the lower sixth of the animals in the Jersey Registry of Merit. The high butterfat testing group consisted of 446 cows having a butterfat test above 5.9 percent. These cows represented the highest testing animals in the Jersey Registry of Merit. They, too, constituted about a sixth of the animals tested and were from tested dams. The pedigrees of these animals were run out to three generations. The average mature-form butterfat percentage of the registry of merit daughters of each individual in these pedigrees was determined. These records were then averaged for each ancestral position in the pedigree. Besides this average, the average butterfat percentage of each ancestral group was determined for the female line of the pedigrees.

Low Butterfat Percentage

Daughters' butterfat percentage under 4.9 percent.	Sire; daughters' average butterfat percentage 5.18.	Paternal grandsire; daughters' average butterfat percentage 5.32.
		Paternal granddam; daughters' average butterfat percentage 5.26, average paternal granddam's butterfat percentage 5.16.
	Dam; daughters' average butterfat percentage 4.99, average dam's butterfat percentage 4.96.	Maternal grandsire; daughters' average butterfat percentage 5.22.
		Maternal granddam; daughters' average butterfat percentage 5.12, average maternal granddam's butterfat percentage 5.15.

High Butterfat Percentage

Daughters' butterfat percentage above 5.9 percent.	Sire; daughters' average butterfat percentage 5.51.	Paternal grandsire; daughters' average butterfat percentage 5.41.
		Paternal granddam; daughters' average butterfat percentage 5.42, average paternal granddam's butterfat percentage 5.49.
	Dam; daughters' average butterfat percentage 5.75, average dam's butterfat percentage 5.64.	Maternal grandsire; daughters' average butterfat percentage 5.42.
		Maternal granddam; daughters' average butterfat percentage 5.56, average maternal granddam's butterfat percentage 5.55.

These typical pedigrees for cows of low butterfat percentage and for cows of high butterfat percentage display essentially the same general features as those for milk yield. Throughout, the ancestors of the pedigree of the low butterfat tests have lower butterfat-testing daughters than those of the high butterfat tests. Within either group the records of the parents are more nearly like those of their offspring than are the records of the grandparents. The butterfat percentages of the four grandparents are essentially the same within each group. Consequently no selective breeding for butterfat percentage appears to have been practiced in the Jersey breed.

The data for the actual average butterfat percentages of the female ancestors in the pedigree show the importance of the ancestors closest to the animal under study as contrasted with the relative lack of importance of those a generation further removed. The average dams' butterfat percentage of the low-producing line was 4.96 percent, whereas that of the average dam in the high-producing line was 5.64. The grandparents, on the other hand, show for the low-producing line 5.16 and 5.15 percent as their averages, whereas those of the high-producing line were 5.49 and 5.55 percent. From other evidence it has been possible to show that the resemblance one generation further removed (the great grandparents) is even less. The data show that no significant selection of bulls to be used as sires has been made on the basis of their dams' butterfat percentage. The butterfat percentages of the paternal granddams and of the maternal granddams seem for practical purposes to be the same.

The pedigrees here shown graphically illustrate the effects of inheritance and environment on the milk yield or butterfat percentage of particular groups of animals. The problem before us is to analyze these two variables in such a way as to be able to ascribe to each ancestor and to the environment its contribution to the total effect.

The contribution of the particular ancestor to the cow's whole inheritance for milk production is ascertained by comparing the production of the cow with that of her parents, sisters, cousins, and grandparents. The environmental effects are determined by comparing the production of individuals of the same generation within the same herd with that of other generations. The form in which the results are presented is designed to indicate the basic information worthy of consideration in practical breeding as well as the relative importance of the different ancestors in the strict genetic sense.

Influence of Sire on Milk Production and Butterfat Percentage

The most frequently used measure of the sire's influence on the milk production of his progeny is the average production of his daughter. This measure is based on the assumption that if milk yield is truly inherited the expectation would be that the sire's daughters would show the type of production which he transmitted to them. His daughters would be expected to resemble each other more than cows of the general breed because of the common inheritance they received from him. If the daughters were full sisters then the resemblance between their productions would be still greater, for here both parents would contribute to the like inheritance. There are 1,830 sires with 2 or more daughters in the Jersey Registry of Merit. The comparison of the milk yields and butterfat percentages of the 110,456 pairs of half sisters sired by these sires gives the data in table 1:

TABLE 1

Comparison of Milk Yield (Pounds) and Butterfat Percentage of 110,456 Pairs of Half Sisters Sired by the 1,830 Sires with 2 or More Daughters in the Jersey Registry of Merit

Item	Milk-yield data	Butterfat percentage data
Average record	10,065±17	5.37±0.003
Standard deviation	2,470±12	0.515± .003
Coefficient of variation	24.5	9.6
Correlation coefficient	0.24±0.01	0.25± .01

If the average production of these half sisters is compared with that found for the breed as a whole at mature form, 9,849 pounds of milk and 5.31 per cent of butterfat, it will be seen that these half sisters are slightly above the breed's average production in milk and practically average in butterfat percentage. The variation in the milk yield is somewhat more than the breed's average (2,254 pounds), while the variation in butterfat percentage is representative (0.516). The correlation coefficients show that there is a fairly well-marked relation between the milk yields or the butterfat percentages of these half sisters. This relation shows the influence of the sire on his daughters' producing capacity since these daughters are from different dams, each dam having a different heredity. The linear equations expressing the relation between the production of one half sister and that of another as determined from the foregoing values are:

Half sister's average milk yield = 7,719 + 0.23 half sister's milk yield

Half sister's average butterfat percentage = 4.03 + 0.25 half sister's butterfat percentage

Table 2 is based on these equations and illustrates the probable or average production of a cow, half sister to a cow of known production. Thus if the production of the half sister is 7,000 pounds the probable production of another cow by the same sire but by a different dam would be 9,350 pounds. Where one of the sisters has a milk yield of 19,000 pounds, the milk yield of the other tends to be less, 12,146 pounds. The average yields tend to regress toward the average value of the breed when the first half sisters are extremely low or extremely high producers. The same is true for the butterfat percentages.

TABLE 2

Average Milk Yield (Pounds) and Butterfat Percentage of Half Sisters from a Given Sire

Actual milk yield of first daughter	Average milk yield of half sisters	Actual butterfat percentage of first daughter	Average butterfat percentage of half sisters	Actual milk yield of first daughter	Average milk yield of half sisters	Actual butterfat percentage of first daughter	Average butterfat percentage of half sisters
5,000	8,884	3.5	4.90	13,000	10,748	5.5	5.40
7,000	9,350	4.0	5.03	15,000	11,214	6.0	5.53
9,000	9,816	4.5	5.15	17,000	11,680	6.5	5.65
11,000	10,282	5.0	5.28	19,000	12,146	7.0	5.77

In breeding practice this type of problem is presented in a variety of ways. It occurs most frequently in a case where the first offspring of a sire has a low record, say 7,000 pounds of milk, and the owner of the sire is considering the advisability of discontinuing his further use. Table 2 shows that a second daughter by this sire would have a probable production of 9,350 pounds of milk. While this is not high, it is better than the 7,000 pounds produced by the first daughter.

The data in table I give the information necessary to determine not only the probable production of a bull's daughter, but also the amount of variation which they would be expected to exhibit. The probable production of the bull's daughters determined from that of their half sisters is given in the equation presented above. The amount of variation which the bull's daughters will have around their average production is determined by the correlation coefficient and the standard deviation. This variation is equal to the standard deviation as found in table 1 (2,470 for milk and 0.515 for butterfat percentage) multiplied by the square root of 1 minus the correlation coefficient squared, $S.D. \sqrt{1 - r^2}$. The value of the correlation coefficient for the milk yield is 0.24 and for the butterfat percentage 0.25. The standard deviation expected in the bull's daughters' production is therefore 2,397 pounds for milk yield and 0.499 percent for the butterfat percentage. Fifty percent of the daughters would be expected to be within $0.67449 \times 2{,}397 = 1{,}618$ pounds on either side of the average production of the group, assuming a normal distribution. Thus if a half sister's production is 9,000 pounds the rest of the bull's daughters would be expected to have an average production of 9,816 pounds. Fifty percent of these daughters would be expected to produce between $9{,}816 - 1{,}618 = 8{,}198$ and $9{,}816 + 1{,}618 = 11{,}434$ pounds of milk. In the same manner it is possible to determine the extreme limits of the production. The limit of the distribution from the average value, 9,816 pounds, to include 99 percent of the daughters is 2.58 times the standard deviation on either side of the mean. In actual figures this limit would be $2{,}397 \times 2.58$, or 6,192 pounds on either side of the average value. For example, if a bull's first daughter produced 15,000 pounds of milk the expected average production of his other daughters would be 11,214 pounds; 50 percent of the daughters would produce between 9,596 and 12,832 pounds, and 99 percent would produce between 5,022 and 17,406 pounds. These facts bring out a significant point. While it is possible to estimate the average production of these half sisters, there still remains a wide variation within which the individuals half sisters' production may vary, making it impossible to determine the actual production of a single half sister exactly when the only information available is that of a half sister's production.

The degree of resemblance existing between the milk yields or butterfat percentages of half sisters in other breeds of dairy cattle is of some importance since it strengthens any belief that may be held as to the real nature of the facts observed for the cows within the Jersey herd. The correlation between the milk yields of half sisters with a common sire was 0.24 for the Jersey Registry of Merit cows, 0.36 for the advanced registry Holstein-Friesian cows, and 0.13 for the advanced registry Guernsey cattle. For the butterfat percentages the correlations for these Jerseys was 0.25, for the Holstein-Friesian 0.37, and for the Guernsey 0.17 (*11*, *13*, *14*). The range of values for the different breeds is perhaps greater than would be expected. The results agree, however, in showing that the sire's progeny tend to resemble each other to a noticeable degree, enough so that their records may be used advantageously in pedigree breeding. The average degree of likeness is such as might be expected were the breeding at random so far as the inheritance factors for milk yield or butterfat percentage are concerned and the inheritance by the multiple-factor Mendelian scheme.

Ordinarily the influence of a parent on the progeny is determined by a comparison such as the size of the sire with the size of his daughters. Unfortunately this direct method of comparison is not available in the study of inheritance in a character expressed only by one sex. The correlation may be determined by the following somewhat indirect method. The daughters of a single sire would in any case all fall within an array of a correlation table. In normal correlation the constants of such an array are related to those of the total array in such a manner that the standard deviation of an array within the table is equal to the standard deviation of the whole population multiplied by $\sqrt{1 - r^2}$ when r is the correlation sought.

This correlation, while tedious to calculate, makes it possible to determine the relation between two variables, in this case sire and daughter, where only one can be measured. The method is subject to 1 or 2 assumptions and selective factors which should be recognized in the interpretation of the observed facts. In the first place, the correlation surface should be normal. Unfortunately this point cannot be determined, although it is a fair assumption that it is approximately normal since the tables of daughter with dam, etc., are. The array standard deviations are calculated around the rough observational means. If these means do not lie close to a straight line corresponding to the regression line, then the correlation coefficient found by this method is larger than the product moment *r*. Again, in determining the standard deviation of an array, the number of individuals available materially affects its size. This difficulty may in part, and possibly entirely, be overcome by the use of the corrections of the standard deviations of small samples as worked out by Student (*1*), Fisher (*7*), and others (*2*, *30*). The probable errors of the correlation coefficients calculated in this matter are partly dependent on the number of classes and are fairly large. They are not well worked out.

There are 208 sires with 10 or more daughters on which standard deviations have been calculated. In view of the volume of the data it seems inadvisable to list these individually. Each standard deviation was corrected for the influence of the number of daughters as indicated above and the mean obtained, 1,905 pounds for milk yield and 0.443 for butterfat percentage. The standard deviation of the general population is 2,470 pounds for milk and 0.515 for the butterfat percentage. The correlation for milk yield is 0.64 and for butterfat percentage 0.51. On the basis of Mendelian inheritance and random mating these correlation coefficients are somewhat larger than the expected 0.5. However, there are two factors which would tend to make this difference. The ancestry of the daughters on the dam's side is not random, for a fair proportion of the daughters of a sire have common dams as well as sires, which tends to increase the correlation coefficients. The common environment, feeding, care, and management of these cows also serve to make them more alike than they would be solely through inheritance. This factor has a more pronounced effect in these data than it would have in any ordinary correlation table since so much depends on the single variable, daughter's production. We can therefore discount somewhat the numerical value of these correlations between the sire and daughter when making quantitative comparisons with other data. The fact is clear, however, that qualitatively the sire exerts a very decided influence on the quantity of the milk yield and the butterfat percentage of his daughters' daughters.

Joint Influence of Sire and Dam on Milk Production and Butterfat Percentage

Full sisters with the same sire and dam would be expected to have milk yields and butterfat percentages more nearly alike than would half sisters with only a common sire. The Jersey Registry of Merit cows show just such a difference. There are 1,924 pairs of full sisters in these Jersey records—618 sets of 2, 78 of 3, 15 of 4, and 2 of 5. A comparison of the records of one full sisters with that of another is shown in table 3.

The full sisters are essentially the same as the other cows in the Registry of Merit in their mean production of milk and percentage of butterfat. The standard deviation of the milk yields and butterfat percentages, are like those of the other cows. The correlation coefficients show a well-marked relation between the milk yields and butterfat percentages of these full sisters. This relation is much more pronounced than that found for any item of the cow's own conformation correlated with her milk yield or butterfat percentage. It is greater than that for half sisters with a common sire. As the sire's influence was shown by the likeness of the half sister's milk and butterfat production, so the sire's and dam's influence acting jointly is shown by these full-sister records. In view of the fact that the environment of half sisters is like that of full sisters, it follows that this difference can be accounted for only as the contribution of the heredity of the dam toward making these half sisters alike in their milk secretion. The facts consequently warrant the conclusion that both the sire and dam contribute jointly to the cow's inheritance for milk production and butterfat percentage.

TABLE 3

Comparison of Milk Yield (Pounds) and Butterfat Percentage of 1,924 Pairs of Full Sisters, from the Records of the Jersey Registry of Merit

Item	Milk-yield data	Butterfat percentage data
Average record	10,114±43	5.34±0.01
Standard deviation	2,482±30	0.511 ± .006
Coefficient of variation	24.6	9.57
Correlation coefficient	0.39±0.01	0.41± .01

From the foregoing data the relation of the production of full sisters may be put into equation form:

Full sister's milk yield = 6,210 + 0.386 other full sister's milk yield

Full sister's butterfat percentage = 3.18 + 0.405 other full sister's butterfat percentage

As a guide in breeding operations these equations have the same utility as those of the half sisters with a common sire. The joint effect of the sire and dam is strikingly evident in table 4, where the solution of these equations is presented for certain productions. From these equations, the reader can obtain any expected production of full sisters that may be desired. When the production of the first full sister corresponds with any of those given in the table the same answers should be obtained as are given therein. Should the milk yield of a cow be 10,000 pounds the expected milk yield of a full sister would be 10,000 × 0.386 + 6,210 = 3,860 + 6,210 = 10,070 pounds. In like manner the butterfat percentages are also obtained.

TABLE 4

Average Milk Yield (Pounds) and Butterfat Percentage of Full Sisters

Actual milk yield of first daughter	Average milk yields of full sisters	Actual butterfat percentage of first daughter	Average butterfat percentage of full sisters	Actual milk yield of first daughter	Average milk yields of full sisters	Actual butterfat percentage of first daughter	Actual butterfat percentage of full sisters
5,000	8,140	3.5	4.59	13,000	11,228	5.5	5.40
7,000	8,912	4.0	4.80	15,000	12,000	6.0	5.61
9,000	9,684	4.5	5.00	17,000	12,772	6.5	5.81
11,000	10,456	5.0	5.20	19,000	13,542	7.0	6.01

The variation in the productions of individual cows around that expected of full sisters as seen in table 4 may be determined in the same manner as those derived for the sire. The 50-percent range is found to be ± 1,531 pounds for milk yield and ± 0.314 for butterfat percentage. The 99-percent range for milk yield is ± 5,895 and for butterfat percentage ± 1.203.

The full-sister correlations for these Jersey cows were 0.39 for milk yield and 0.41 for butterfat percentage. Similar correlations have been determined for the Holstein-Friesian and the Guernsey breeds. These correlations were, for the Holstein-Friesian, 0.55 ± 0.03 for milk yield and 0.46 ± 0.03 for butterfat percentage; for the Guernsey, 0.41 ± 0.02 for milk yield and 0.44 ± 0.02 for butterfat percentage. The data for these three breeds are consequently not very different. Furthermore they are in accord with the hypothesis that the sire and dam are jointly responsible for the milk production and butterfat percentage of their offspring. The data are also in fair accord with the expectation of theoretical inheritance when it is assumed that the character of the milk secreted by a cow is determined by multiple Mendelian factors and that the population is random bred, as such assumptions lead to an expected correlation between full sisters of 0.5 and between half sisters of 0.25. The actual averages are, for full sisters, 0.44 for milk yield and 0.45 for butterfat percentage, and, for half sisters with a common sire, 0.24 for milk yield and 0.26 for butterfat percentage.

Influence of Dam on Milk Production and Butterfat Percentage

The influence of the dam on the milk yield of her progeny may be measured in at least two ways—by determining (1) the relation which exists between the records of half sisters when the dam is the common parent, and (2) the correlation between the milk produced by the dam and that produced by her daughter. The data on the milk yield of half sisters with a common dam are presented first.

There are 1,603 of these sisters groups—1,248 sets of 2, 248 of 3, 80 of 4, 17 of 5, 5 of 6, and 5 of 7, making a total of 5,644 pairs. Of this total, 1,924 pairs are full sisters and must be subtracted, leaving 3,720 pairs in all. The constants for measuring the relation between these half sisters are shown in table 5.

The half sisters with a common dam produce slightly less milk than do either the half sisters with a common sire or full sisters. The butterfat percentage of the three groups is practically identical. The variation in milk yield is somewhat less than that for the other two groups. It is, however, in line with the mean production in that it is reduced, thus tending to make the coefficients of variation of the three groups the same. The variation in butterfat percentage is practically identical with that of the other groups both absolutely and in percentage. Characteristically the coefficient of variation in milk yield is two and one-half times as much as that for butterfat percentage, indicating that milk yield is relatively much more variable than is the proportion of its butterfat.

The correlation coefficients are, of course, the most important feature of table 5 from the breeding point of view. The correlations between half sisters of the two kinds (sire or dam in common) are distinctly comparable if not exactly the same. Such being the case it may be concluded that the sire and dam are equally important in determining the quantity of milk produced as well as the percentage of butterfat of their progeny. This conclusion is further borne out by the fact that when the joint influence of the sire and dam is measured the correlations are double those obtained when one is measured separately, as we should expect if the sire and dam contribute equally to the progeny's genetic constitution for milk production or butterfat percentage.

TABLE 5

Comparison of Milk Yield (Pounds) and Butterfat Percentage of 3,720 Pairs of Half Sisters with a Common Dam, from the Records of the Jersey Registry of Merit

Item	Milk-yield data	Butterfat percentage data
Average record	9,882±34	5.36 ±0.01
Standard deviation	2,373±25	.510± .01
Coefficient of variation	23.9	9.5
Correlation coefficient	0.19±0.01	.20 ± .01

These facts are of interest from a slightly different point of view, namely, the relative influence of heredity and environment (care, management, etc.) in determining the milk yield or butterfat percentage of the cow. It seems entirely possible that with any such group of cattle as these the milk yield and butterfat percentage of half sisters would be as much influenced by their common environment as those of full sisters. If such is the case, then the difference in the correlations of half sisters and full sisters should measure the extra effect of the common sire or common dam directly attributable to inheritance. The differences between these half and full sisters are on the order of 0.18, which would leave but little remaining as the influence of the common environment. This point is discussed later.

The equations for the relations between the milk yields and butterfat percentages of these half sisters from a common dam are:

Half sister's milk yield = 7,955 + 0.195 other half sister's milk yield

Half sister's butterfat percentage = 4.28 + 0.201 other half sister's butterfat percentage

Table 6 is based upon these equations, expressing the relation of the half sisters' production for a given production of the cow's first daughter.

Table 6 shows that half sisters with a common dam resemble each other as closely in their milk yield and butterfat percentage as do half sisters with a common sire. The likeness of either set of half sisters is not so close as that of full sisters. In numerical terms the data show that where one sister has a production of 5,000 pounds her half sister by the same sire would be expected to have a production of 8,884 pounds, her half sister from the same dam 8,930 pounds, and her full sister 8,140 pounds. The half sisters have practically the same expected milk yields. The full sisters are 760 pounds nearer in their production than half sisters with a common sire or half sisters with a common dam. A similar situation exists where the production of the first cow is high. For a cow with a production of 19,000 pounds the half sisters would be expected to produce about 12,000 pounds, the full sisters 13,542 pounds. Again the full sisters tend to be more alike in their production than half sisters. Similar relationships are observed for the butterfat percentage of half and full sisters.

TABLE 6

Average Milk Yield (Pounds) and Butterfat Percentage of Half Sisters from a Common Dam

Actual milk yield of first daughter	Average milk yield of half sisters	Actual butterfat percentage of first daughter	Average butterfat percentage of half sisters	Actual milk yield of first daughter	Average milk yield of half sisters	Actual butterfat percentage of first daughter	Average butterfat percentage of half sisters
5,000	8,930	3.5	4.98	13,000	10,490	5.5	5.39
7,000	9,320	4.0	5.08	15,000	10,880	6.0	5.49
9,000	9,710	4.5	5.18	17,000	11,270	6.5	5.59
11,000	10,100	5.0	5.29	19,000	11,660	7.0	5.69

The standard deviations for the production of the half sisters, when their average record is that indicated by the equation, are 2,298 pounds of milk and 0.5 percent of butterfat. The ranges necessary to include 50 percent and 99 percent of the half sisters on either side of this average value are 1,551 and 5,935 pounds for milk yield and 0.337 and 1.291 for butterfat percentage.

If the three important dairy breeds, Holstein-Friesian, Guernsey, and Jersey, are compared we find the following correlations between the half sisters with common dams: Milk yield Holstein-Friesian 0.38 ± 0.03, Guernsey 0.15 ± 0.02, and Jersey 0.19 ± 0.01; butterfat percentage Holstein-Friesian 0.22 ± 0.04, Guernsey 0.19 ± 0.01, and Jersey 0.20 ± 0.01. The data for the three breeds are alike in showing a notable resemblance between the production of half sisters with a common dam. Full sisters resemble each other more closely in their milk yield and butterfat percentage than half sisters. The half sisters from a common sire or from a common dam have practically the same degree of resemblance. The hypothesis that the sire and dam are equally and jointly responsible for the milk production and butterfat percentage of their offspring is consequently borne out by this comparison. Furthermore the results are in accord with the expectation of a random-bred Mendelian population. For such a population the relation between full sisters should be measured by a correlation coefficient of 0.5, and between half sisters by a correlation coefficient of 0.25. Actually for the three breeds averaged together the correlation coefficient for the milk yields of full sisters was 0.45 and that for half sisters 0.24. For butterfat percentage the correlation coefficient was 0.43 for full sisters and 0.23 for half sisters.

Relation between Milk Yield and Butterfat Percentage of Dams and Daughters

Table 7 shows the relation between the milk production and butterfat percentage of daughter and dam. The average milk yield and butterfat percentage of the daughters is given for different groups of dams, progressing from low-producing to high-producing dams. There are 3,753 pairs of these daughter and dam records.

The average daughters' production for grades of production of the dams show that as the dams' milk production increases the average daughters' production also increases. These increases show some irregularities since the figures presented are the actual average production and not just the trends as given previously. This average production of the daughters as determined from the trend of these milk yields shows that for 1,000 pounds increase in the milk yield of the dam, the daughter's average increases 316 pounds. In other words, the increase of the daughters is about one-third that of the dams.

The results for butterfat percentage are similar. Dams of higher butterfat percentage tend to have daughters that are high in butterfat percentage, and dams whose butterfat percentage is low tend to have daughters whose butterfat percentage is relatively low. Here, again, increase of the butterfat percentage of the dam causes only a fractional increase in the butterfat percentage of the daughter. The average increase of the daughter's butterfat percentage for a 1 percent increase of the dam's is 0.42 percent.

TABLE 7

Relation between Daughters' and Dams' Average Milk Yield (Pounds) and Butterfat Percentage in 3,753 Pairs of Records

Number of daughter-dam pairs	Dam's milk yield	Daughter's milk yield	Number of daughter-dam pairs	Dam's butterfat percentage	Daughter's butterfat percentage	Number of daughter-dam pairs	Dam's milk yield	Daughter's milk yield	Number of daughter-dam pairs	Dam's butterfat percentage	Daughter's butterfat percentage
12	5,550	9,830	2	3.5	4.20	64	15,500	11,330	415	5.7	5.50
200	6,500	8,900	1	3.9	4.70	36	16,500	12,140	300	5.9	5.62
578	7,500	9,240	35	4.1	4.80	15	17,500	11,500	180	6.1	5.64
727	8,500	9,540	89	4.3	4.96	9	18,500	12,060	81	6.3	5.78
772	9,500	9,930	191	4.5	5.02	3	19,500	9,500	48	6.5	5.82
516	10,500	10,300	277	4.7	5.04	3	21,500	17,200	19	6.7	5.86
329	11,500	10,510	442	4.9	5.16				13	6.9	5.84
255	12,500	11,190	545	5.1	5.26				2	7.1	5.20
159	13,500	11,460	604	5.3	5.34				1	7.9	5.70
75	14,500	10,990	508	5.5	5.44						

The average milk yield and butterfat percentage of the daughters are both larger than those of their dams. The variation of the milk yields of these daughters is also somewhat greater than that of their dams. The correlation coefficients for the production records of daughter and dam are both significant and of medium value. The constants are given in table 8.

The fact that the daughters' production is somewhat higher than that of their dams is a matter of some moment if it truly represents an increase in yield due to better heredity of the daughters or to a better understanding of nutrition. Such a change would indicate real progress. Unfortunately the differences cannot be directly attributed to these causes, for the increase in production is also subject to other interpretations. The animals tested for Registry of Merit might come from a more highly culled class than their dams, a culling based on previous production records. Such a culling would account for the small difference observed.

TABLE 8

Comparison of Milk Yield (Pounds) and Butterfat Percentage of Daughters and Their Dams, from the Records of the Jersey Registry of Merit

Item	Milk-yield data		Butterfat percentage data	
	Daughter	Dam	Daughter	Dam
Average record	10,028±27	9,852±25	5.35 ±0.01	5.31 ±0.01
Standard deviation	2,420±19	2,285±18	.513± .004	.518± .004
Coefficient of variation	24.1	23.2	9.6	9.8
Correlation coefficient	0.30±0.01		0.42±0.01	

The relation between the milk yields and the butterfat percentages of daughters and dams is shown graphically in figures 1 and 2. The irregular line indicates the actual average production of daughters for a given production of dams as shown in table 7. The straight lines represent the average trend of the data as derived from the regression equations.

$$\text{Daughters' milk yield} = 6{,}920 + 0.32 \text{ dams' milk yield}$$

$$\text{Daughters' butterfat percentage} = 3.12 + 0.42 \text{ dams' butterfat percentage}$$

The standard deviations and coefficients of variation of milk yield and butterfat percentage are well within the range of those ordinarily found for dairy cattle. The variation of the butterfat percentage is proportionately only two-fifths that of the milk yield.

The correlation coefficients for milk yield and butterfat percentage of daughter and dam show that inheritance plays an important part in milk secretion. The degree of relationship is lower for milk yield than for butterfat percentage as determined for these data. A comparison of these results with those from other breeds shows that such a difference does not always occur. The correlation coefficients for the milk yield and butterfat percentage of Holstein-Friesian cattle are

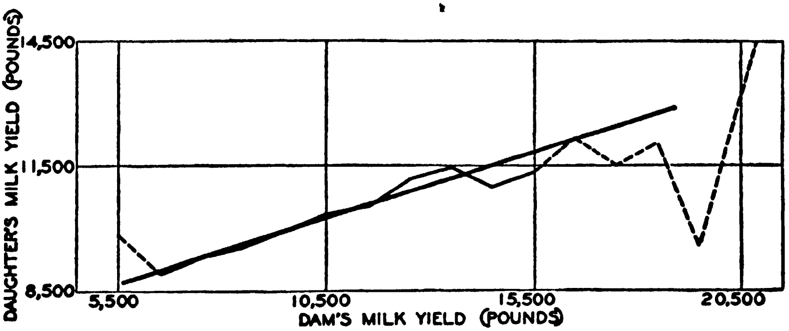

FIG. 1.—Relation between the milk yields of daughter and dam. Straight line represents average trend of the data; solid line connects averages established by large volume of data and broken line those established by few data.

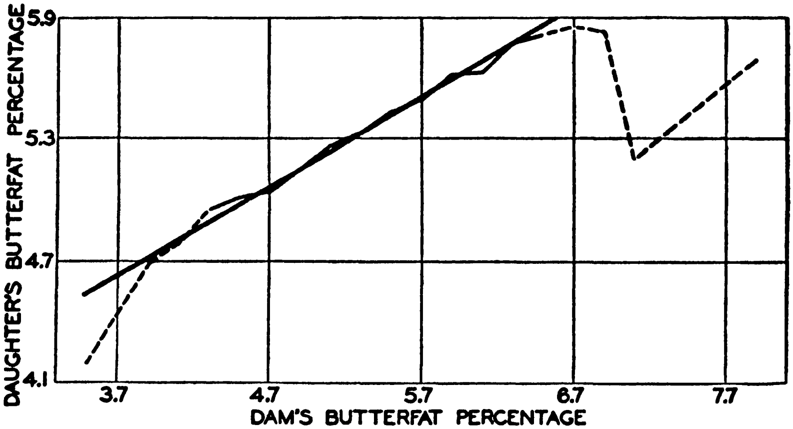

FIG. 2.—Relation between the butterfat percentages of daughter and dam. Straight line represents general trend of the data; solid line connects averages established by large volume of data and broken line those established by few data.

0.50 ± 0.02 and 0.41 ± 0.02, respectively. The difference is in a direction opposite to that found for the Jerseys. The relation between the milk yield and butterfat percentage of daughter and dam in the Guernseys is in the same direction as that for the Jerseys, 0.36 ± 0.02 for milk yield and 0.42 ± 0.02 for butterfat percentage.

The Jersey Registry of Merit cows show a pronounced negative correlation between the milk yield of the cow and the butterfat percentage of her milk, the larger the milk yield the lower the butterfat test of the milk. This correlation is equal to −0.29 ± 0.01 for the daughters and −0.27 ± 0.01 for the dams. A slight negative correlation would therefore be expected between the production of the daughters and the butterfat percentage of the dams brought about by this physiological relationship. Such zero-order correlations are actually found; daughter's milk yield with dam's butterfat percentage equals −0.12 ± 0.01 and daughter's butterfat percentage with dam's milk yield equals −0.11 ± 0.01. If these correlations are determined with the proper daughter's and dam's production or butterfat percentage held constant, the correlations are less in value. The correlation between daughter's milk yield and dam's butterfat percentage for a constant butterfat percentage of the daughter and a constant milk production of the dam equals 0.08 ± 0.02, and the correlation between the daughter's butterfat percentage and the dam's milk yield for a constant daughter's milk yield and dam's butterfat percentage equals 0.08 ± 0.02, values which are scarcely significant. The results are thus comparable with those of the Holstein-Friesian breed.

Relation between Milk Yield and Butterfat Percentage of Grandparents and Granddaughters

Two individuals may have none, any, or all of their grandparents in common. In 1 combination they may have all grandparents in common; in 4 combinations they may have 3 grandparents in common; in 6 combinations, 2, in 4 combinations 1, and in 1 combination none—16 cousin combinations in all. Within a single class the degree of genetic correlation will also vary. Those individuals with 4 common grandparents may have these as the result of a repeated mating of the same sire and dam, when they will be full sisters, matings of the same sire to full sisters, matings of full brothers to the same cow, or matings of full brothers and sisters. On the basis of multiple-factor inheritance for a character and random segregation, the expected genetic correlations for such individuals are distinctly different.

There are equally significant differences in the other ancestral groups. So far as possible an attempt is made to separate these genetically different groups. The data of table 9 show the correlations between the production of the granddaughters for the different combinations of common grandparents. The paternal grandsire is represented by A, the paternal granddam by B, the maternal grandsire by C, and the maternal granddam by D. The first column of table 9 shows the number of common grandparents for the particular cows whose milk yield or butterfat percentage is correlated. The records are presented for individuals having 4, 3, 2, and 1 common grandparent. The other possible combinations of grandparents had so few pairs of granddaughters for comparison that they are omitted from the table. The A C D group, for example, had but 66, the B C D but 60, etc.

The comparison of milk production or butterfat percentage of the pairs of granddaughters shows the largest correlations for the cows that have the largest number of grandparents in common and progressively smaller correlations as the number of common grandparents diminishes. When the cows have four grandparents in common because of having had the same sire and dam their milk yields or butterfat percentages are most nearly alike, as indicated by the correlations 0.39 and 0.41. The next highest correlations are found where the cows have three grandparents in common, the paternal grandsire and granddam being common to both because the sire was the same bull. Following these in the degree of their relationship are the cows which have all four grandparents in common, the sires of these cows being brothers and the dams sisters. The cows which have but two grandparents in common show a still smaller correlation between milk yields or butterfat percentages. Cows with but one common grandparent show the smallest relation between their productions. The results of this study are consequently what would be expected for an inherited character, namely, that the greater the number of common ancestors the greater the degree of resemblance between the related individuals.

TABLE 9

Correlation between the Milk Yield (Pounds) or Butterfat Percentage of Individual Cows Having the Indicated Relationship

Grandparents common[1]	Parents common	Number of pairs	Correlation coefficients		Mean		Standard deviation		Prediction equations[2]		Variation range to include—			
											50 percent		99 percent	
			Milk yield	Butterfat percentage	Milk yield	Butterfat percentage	Milk yield	Butterfat percentage	Milk yield	Butterfat percentage	Milk yield	Butterfat percentage	Milk yield	Butterfat percentage
A B C D....	Sire and dam.	1,924	0.39	0.41	10,114	5.34	2,482	0.51	6,170+0.39 M	3.15+0.41 F	1,541	0.31	5,895	1.20
A B C D....	....	474	.26	.28	9,846	5.38	2,450	.51	7,286+ .26 M	3.87+ .28 F	1,596	.33	6,104	1.26
A B C.......	Sire.........	14,426	.31	.29	10,161	5.38	2,676	.51	7,011+ .31 M	3.82+ .29 F	1,716	.33	6,564	1.26
A B D.......	.....do......	720	.30	.24	10,727	5.42	2,286	.55	7,509+ .30 M	4.12+ .24 F	1,471	.36	5,627	1.38
A B.........	.....do......	99,458	.24	.25	10,057	5.37	2,463	.51	7,643+ .24 M	4.03+ .25 F	1,613	.33	6,169	1.27
A B.........	....	4,612	.25	.27	10,128	5.38	2,787	.37	7,596+ .25 M	3.93+ .27 F	1,820	.24	6,963	.92
A and C are same bull..	....	1,374	.12	.24	9,651	5.48	2,145	.48	8,493+ .12 M	4.16+ .24 F	1,437	.31	5,495	1.20
A C.........	....	1,566	.15	.10	10,024	5.38	2,579	.50	8,520+ .15 M	4.84+ .10 F	1,720	.34	6,579	1.28
B C.........	....	948	.35	.14	11,235	5.43	2,870	.41	7,303+ .35 M	4.67+ .14 F	1,813	.27	6,935	1.05
C D.........	Dam........	3,720	.19	.20	9,882	5.36	2,373	.51	8,004+ .19 M	4.29+ .20 F	1,572	.34	6,011	1.29
C D.........	....	1,656	.23	.22	9,794	5.39	2,393	.50	7,541+ .23 M	4.20+ .22 F	1,571	.33	6,009	1.26
A...........	....	489,526	.02	.07	9,750	5.34	2,322	.52	9,555+ .02 M	4.97+ .07 F	1,566	.35	5,991	1.34
B...........	....	26,028	.06	.07	10,414	5.34	2,464	.58	9,789+ .06 M	4.97+ .07 F	1,659	.39	6,347	1.49
C...........	....	64,150	.12	.04	9,827	5.36	2,386	.50	8,648+ .12 M	5.15+ .04 F	1,598	.34	6,112	1.29
D...........	....	3,198	.15	.04	9,773	5.37	2,373	.54	8,307+ .15 M	5.16+ .04 F	1,582	.36	6,053	1.39

[1] A = paternal grandsire; B = paternal granddam; C = maternal grandsire; and D = maternal granddam.

[2] M or F = Milk yield or butterfat percentage of cow having indicated relationship to the first.

The number of pairs having the common ancestors shown in table 9 is enlightening. There are 609,676 possible pairs of cows in these data having a common paternal grandsire, A, [illegible]2 pairs having a common paternal granddam, B; 89,764 cows having a common maternal grandsire, C; and 11,374 having a common maternal granddam, D. For the first generation there are 112,390 pairs of cows with common sires to 5,644 with common dams. The notably greater distribution of the sire's germ plasm is evident. Of the 609,676 pairs with common paternal grandsires a third had other common grandparents. Of the 144,052 pairs with B in common, 82 percent had other common grandparents. In the maternal line 29 percent of the cows having a common grandsire had other common grandparents, while of the cows with common granddams 72 percent had other common grandparents. Some combinations of grandparents are quite rare. It is unlikely that two cows would have A C D, B C D, A D, or B D in common without having other common grandparents. This uneven distribution of grandparents becomes significant in considering ancestral correlation for milk yield or butterfat percentage, especially when these are obtained by indirect means (ratio of standard deviation of daughters of a single sire to the standard deviation of the whole population), since the chance of the cows having other ancestors repeated besides the sire in question is great. The means and standard deviations of the different ancestral groups vary somewhat. The variation does not appear to be excessive, however.

Paternal Granddam

The effect of the inheritance received from the granddam on the granddaughter's milk secretion may be measured directly by comparing the amount of milk secreted by each pair of granddams and granddaughters. There are 2,709 pairs of paternal granddams and granddaughters for comparison. The data showing the average granddaughters' records for given productions of the paternal granddams are presented in table 10.

The general trend of the granddaughters' production in either milk or butterfat percentage for increasing production of the granddams is toward somewhat larger values. For 1,000 pounds increase in the paternal granddams' milk yield, the granddaughters' milk yield increases 159 pounds. The increase of the granddaughters is consequently about one-sixth that of the granddams. The results for butterfat percentage are similar. In general paternal granddams whose butterfat percentage is high have granddaughters with somewhat higher butterfat tests than do granddams whose butterfat percentage is low. On an average when there is a 1-percent difference in the butterfat percentage of the granddams, the average tests of the granddaughters differ by 0.21 percent. These results are shown in figures 3 and 4. The constants to measure these are shown in table 11.

TABLE 10

Relation between Granddaughters' and Paternal Granddams' Milk Yield (Pounds) and Butterfat Percentage in 2,709 Pairs of Records

Pairs of granddaughter-granddam (number)	Paternal granddam's milk yield	Granddaughter's milk yield	Pairs of granddaughter-granddam (number)	Paternal granddam's butterfat percentage	Granddaughter's butterfat percentage	Pairs of granddaughter-granddam (number)	Paternal granddam's milk yield	Granddaughter's milk yield	Pairs of granddaughter-granddam (number)	Paternal granddam's butterfat percentage	Granddaughter's butterfat percentage
3	5,500	9,500	10	4.1	5.16	157	13,500	11,017	448	5.7	5.44
88	6,500	9,300	87	4.3	5.12	71	14,500	10,424	195	5.9	5.42
252	7,500	9,130	169	4.5	5.06	94	15,500	11,195	122	6.1	5.48
458	8,500	9,479	118	4.7	5.32	82	16,500	11,019	27	6.3	5.56
311	9,500	9,701	372	4.9	5.28	182	17,500	10,815	34	6.5	5.46
433	10,500	10,504	379	5.1	5.28	20	18,500	9,750	19	6.7	5.49
363	11,500	10,173	305	5.3	5.34	28	19,500	10,640	2	6.9	5.91
167	12,500	9,693	422	5.5	5.46						

The supposed changes of domestic breeds of animals since they separated from their wild progenitors have furnished presumptive evidence for the theory of evolution. The presumed changes in the milk yields of dairy cattle have played their part in this argument, a part possibly greater than the facts warrant, for records of production adequate enough for any real selection are a recent acquisition to animal husbandry. It is questionable if prior to our own generation there was any selection of dairy stock for breeding which was concerted enough to be in any sense effective. The production of the paternal granddams when compared with that of the whole Jersey breed and with that of the maternal granddams indicates that selection for larger milk secretion is in progress in the Jerseys. The average milk yield of these paternal granddams is about 1,100 pounds more, that is, one-tenth more than for the average cow in the advanced registry. The evidence thus shows that there has been a real selection of bulls used for breeding on the basis of the milk-production record of their dams. No such selection has been practiced for the butterfat percentage, however, the average butterfat record of the paternal granddam being no higher than that of the breed average. The selective breeding that is actually practiced emphasizes the milk yield. The degree of this selection within the Jersey Registry of Merit ani-

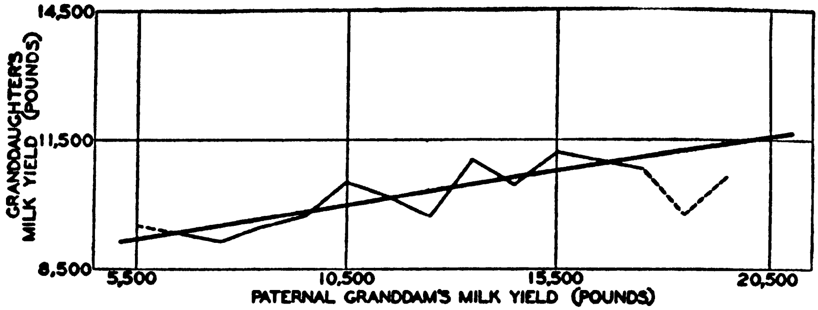

FIG. 3.—Relation between the milk yields of granddaughters and paternal granddams. Straight line represents average trend of the data; solid line connects averages established by large volume of data and broken line those established by few data.

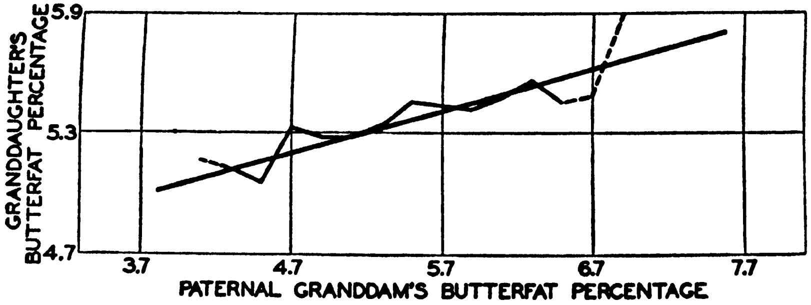

FIG. 4.—Relation between the butterfat percentages of granddaughters and paternal granddams. Straight line represents average trend of the data; solid line connects averages established by large volume of data and broken line those established by few data.

mals is greater than it is with a sister breed, the Holstein-Friesian, as shown by similar studies of this breed.

The foregoing data arranged to give the granddaughters' probable milk secretion when they have paternal granddams of known production is:

Granddaughter's milk yield = 8,286 + 0.16 paternal granddam's milk yield

Granddaughter's butterfat percentage = 4.21 + 0.21 paternal granddam's butterfat percentage

These equations give the mean production of the granddaughters. The variation on either side of this mean necessary to include 50 percent of the granddaughters from granddams of the same yield is 1,619 pounds for the milk yield and 0.34 for the butterfat percentage. To include 99 percent of the granddaughters, the range is 6,185 pounds for the milk production and 1.30 for the butterfat percentage.

TABLE 11

Comparison of Milk Yield (Pounds) and Butterfat Percentage of Granddaughters and Paternal Granddams, from the Records of the Jersey Registry of Merit

Item	Milk-yield data		Butterfat percentage data	
	Granddaughter	Granddam	Granddaughter	Granddam
Average record	10,057±31	11,146±40	5.35 ±0.01	5.32 ±0.01
Standard deviation	2,430±22	3,105±28	.509± .005	.501± .005
Coefficient of variation	24.2	27.9	9.5	9.4
Correlation coefficient	0.20±0.01		0.21±0.01	

Maternal Granddam

The production records of 1,466 granddaughters and the corresponding record for the maternal granddams are shown in table 12. There is a definite increase in milk secretion by the granddaughters as the milk secreted by the maternal granddam increases. This increase is somewhat irregular but the trend is evident. For each 1,000 pounds increase in the granddams' production of milk the granddaughters show an average rise of 155 pounds, or about one-sixth of the granddams' increase. For the butterfat percentage an increase of 1 percent for the granddams results in an increase of 0.26 percent in the average granddaughters' butterfat percentage.

TABLE 12

Relation between Granddaughters' and Maternal Granddams' Milk Yield (Pounds) and Butterfat Percentage in 1,466 Pairs of Records

Granddaughter-granddam pairs (number)	Maternal granddam's milk yield	Granddaughter's average milk yield	Granddaughter-granddam pairs (number)	Maternal granddam's butterfat percentage	Granddaughter's average butterfat percentage	Granddaughter-granddam pairs (number)	Maternal granddam's milk yield	Granddaughter's average milk yield	Granddaughter-granddam-pairs (number)	Maternal granddam's butterfat percentage	Granddaughter's average butterfat percentage
109	[1]7,000	9,210	8	[1]4.2	4.82	50	13,500	10,980	203	5.5	5.43
239	7,500	9,990	27	4.3	5.12	24	14,500	10,540	198	5.7	5.44
302	8,500	9,800	80	4.5	5.24	12	15,500	11,000	123	5.9	5.53
299	9,500	10,310	107	4.7	5.17	7	16,500	11,070	76	6.1	5.60
208	10,500	10,330	166	4.9	5.26	15	17,500	10,570	32	6.3	5.56
128	11,500	10,380	167	5.1	5.32	6	[2]18,000	12,500	33	[2]6.4	5.70
67	12,500	10,250	246	5.3	5.36						

[1] Below.
[2] Above.

TABLE 13

Comparison of Milk Yields (Pounds) and Butterfat Percentage of Granddaughters and Maternal Granddams, from the Records of the Jersey Registry of Merit

Item	Milk-yield data		Butterfat percentage data	
	Granddaughter	Granddam	Granddaughter	Granddam
Average record	10,125±45	9,623±39	5.37 ±0.01	5.35 ±0.01
Standard deviation	2,549±32	2,236±28	.517± .006	.513± .006
Coefficient of variation	25.2	23.2	9.6	9.6
Correlation coefficient	0.136±0.017		0.254±0.016	

The average milk yields and butterfat percentages of the granddaughters are somewhat larger than those of the maternal granddams. The averages and constants of variation for the 1,466 pairs are given in table 13.

The average milk production of the paternal granddams was more than 1,100 pounds greater than the average of all cows that had been tested. The evidence was consequently in line with the belief that the bulls used as breeders came from dams that produced more than the average amount of milk. Such a selection for all animals within the ancestry could conceivably affect the production of milk. It is a significant fact that the average production of the maternal granddams, 9,623 pounds, is, if anything, below average rather than above it. These cows are probably not so far below the breed average as would appear from the figures, for an increase in recorded production has been going on within the Jersey Registry of Merit for some time, owing possibly to better breeding or perhaps to a better understanding of those factors that make for a high test record and to a more stringent selection of the animals that enter the test. Such an increase would make the lower producing maternal granddams, since they would be one decade removed, really average-producing cows for the breed. The conclusion is therefore warranted that the cows selected for paternal granddams are a highly selected group while the maternal granddams are not more than average cows. As the data on the inheritance of production all point to the fact that the sire's side and the dam's side of the pedigree are equally important in determining the milk production of their progeny, and as the maternal granddams are no better than breed average in production, it is apparent that no

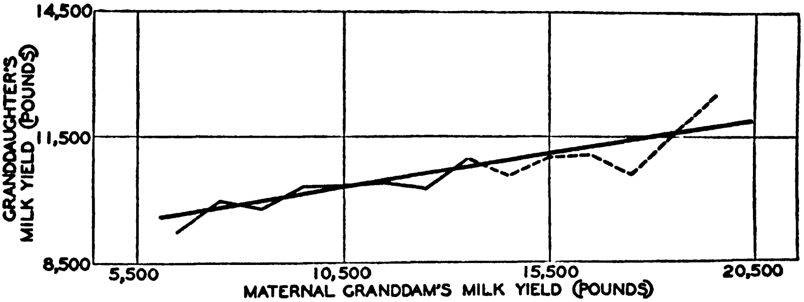

FIG. 5.—Relation between the milk yields of granddaughters and maternal granddams. Straight line represents average trend of data; solid line connects averages established by large volume of data and broken line those established by few data.

selection of the higher producing cows for breeders has been practiced. Good breeding practice calls for a selection of better producing maternal granddams. The butterfat percentages of the granddaughters and the granddams are essentially the same. Furthermore they are practically the same as those found for the average of all the advanced registry. These facts point to the conclusion that the breeding of Jersey cows for butterfat percentage is practically at random.

The average production of the granddaughters for a given production of the maternal granddams is shown graphically in figure 5 for milk yield and in figure 6 for butterfat percentage. The straight lines which represent the average trend of the data are derived from the following equations:

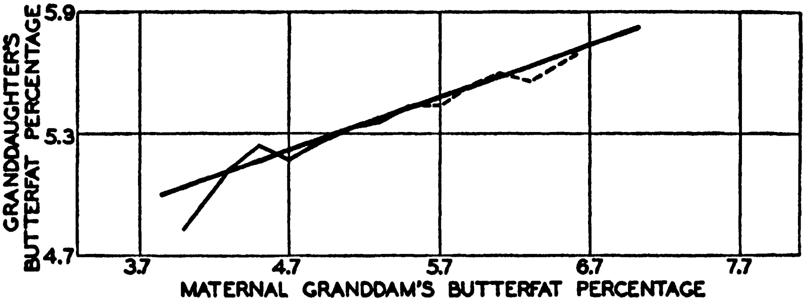

FIG. 6.—Relation between the butterfat percentages of granddaughters and maternal granddams. Straight line represents average trend of data; solid line connects average established by large volume of data and broken line those established by few data.

Granddaughter's milk yield = 8,633 + 0.16 maternal granddam's milk yield

Granddaughter's butterfat percentage = 4.00 + 0.26 maternal granddam's butterfat percentage

These equations, like others presented, simply represent average trends. The variation necessary to include 50 percent of the daughters around this average is 1,722 pounds of milk and 0.35 percent of butterfat on either side of this average value. The range necessary to include 99 percent of the granddaughters having maternal granddams of a single grade of production is 6,539 pounds of milk or 1.33 percent of butterfat on either side of this production.

The relative contributions of the paternal and maternal granddams to the productivity of the granddaughter are important. A comparison of the records of maternal granddams found in this section with those shown in the section on paternal granddams gives the impression that the effects of these two grandparents are essentially alike. The coefficients of correlation for a grandparent and the grandchild for any Mendelian character dependent on multiple factors for its expression would be expected to equal 0.25 if the breeding has been at random within the group of animals studied. The data just presented show that there has been but little selective breeding within the Jerseys so far as the maternal granddams are concerned. The correlation coefficient between the maternal granddam's milk yield and that of the granddaughter is consequently only about half the value that would be expected, 0.136 to 0.25. The value of the correlation coefficient for the butterfat percentages, 0.254, is as expected.

The correlation coefficients for the paternal granddams' and granddaughters' milk yields and butterfat percentages were, respectively, 0.2 and 0.21. The paternal granddams showed a slightly larger correlation coefficient for milk yield and a slightly smaller coefficient for butterfat percentage than did the maternal granddam. The results throughout are of the same general order of magnitude. From these results it may be concluded that the influence of the paternal and maternal granddams on the granddaughters' milk yield and butterfat percentage is of nearly equal value; in other words, the contribution of these animals to the granddaughters' inheritance is practically the same. This conclusion is strengthened by a comparison of the results from the three breeds, Holstein-Friesian, Guernsey, and Jersey. In all cases the two types of grandparents are about equally important. In terms of correlation these relations for granddaughters' and granddams' records are, for milk yield: Paternal granddam, Holstein-Friesian, 0.26 ± 0.04; Guernsey, 0.16 ± 0.07; and Jersey, 0.20 ± 0.01; for the maternal granddam, Holstein-Friesian, 0.31 ± 0.05; Guernsey, 0.20 ± 0.02; and Jersey, 0.14 ± 0.02. Similar results were obtained from a study of the butterfat percentages. For the Holstein-Friesian, Guernsey, and Jersey breeds, the butterfat percentages of the paternal granddams are correlated with those of their granddaughters to the following extent: 0.09 ± 0.04, 0.15 ± 0.07, and

0.21 ± 0.01. The maternal granddams' butterfat percentages are correlated with their granddaughters' as follows: Holstein-Friesian, 0.19 ± 0.05; Guernsey, 0.20 ± 0.02; and Jersey, 0.25 ± 0.02. The average correlations of the grandparents with those of the granddaughters are, for milk yield, 0.21, and for butterfat percentage, 0.18.

If these average correlations for the grandparents are compared with the average correlation of the daughters and their dams as given earlier, 0.45 for milk yield and 0.42 for butterfat percentage, they are seen to be nearly half the value. This is as it should be if the inheritance of the characters milk yield and fat concentration in the milk are dependent upon multiple Mendelian factors assorting at random.

Assortive Mating

With milk and its products occupying such an important place in the economic structure of our agriculture, it would seem that there would be a definite tendency on the part of breeders of dairy cattle to mate like individuals. The actual amount of such assortive mating has an interest to all students of breeding because it measures the effective selection and breeding which is taking place within dairy cattle for a given character. If selection with assortive mating is to be a real force in molding the characteristics of the breed, several points must be kept in mind. First and foremost, the sires to be used in the different herds must, in large part, come from superior parents. As already shown, many of the Jersey sires meet this qualification. Such sires should then have the best mates which the breed affords. Some measure of the amount of this selective breeding may be had by relating the milk yield of the sire's dam with the milk yield of the cows to which he is bred. This information is given in table 14.

There are 1,375 sires whose dams' production is known bred to cows whose own production records are known. In the terminology of this study, since it is based on the daughters, these ancestors have the relation paternal granddam to dam in the pedigrees. The data of table 14 show that there is a fair relation between the production of the paternal granddams and that of the dams. Sires with dams having larger milk yields tend to be bred to cows which are themselves better than average in production. For every 1,000 pounds of milk increase in the paternal granddam's production, there is an increase of 140 pounds in the production of the dams.

The facts are different for the butterfat percentages. There is practically no relation between the paternal granddams' butterfat percentages and those of the dams.

TABLE 14

Relation between Dams' and Paternal Granddams' Milk Yield (Pounds) and Butterfat Percentage in 1,375 Pairs of Records

Dam-paternal granddam pairs (number)	Paternal granddam's milk yield	Dam's average milk yield	Dam-paternal granddam pairs (number)	Paternal granddam's milk yield	Dam's butterfat percentage	Dam-paternal granddam pairs (number)	Paternal granddam's milk yield	Dam's average milk yield	Dam-paternal granddam pairs (number)	Paternal granddam's milk yield	Dam's butterfat percentage
45	[1]7,000	8,830	51	[1]4.4	5.39	82	13,500	10,670	262	5.7	5.39
103	7,500	9,520	84	4.5	5.22	37	14,500	9,580	83	5.9	5.42
246	8,500	9,440	62	4.7	5.37	65	15,500	10,810	61	6.1	5.42
131	9,500	9,770	193	4.9	5.30	42	16,500	10,600	9	6.3	5.59
210	10,500	10,110	188	5.1	5.39	138	17,500	10,790	15	6.5	5.33
165	11,500	10,140	160	5.3	5.38	30	[2]18,000	11,200	10	6.7	5.36
81	12,500	10 230	197	5.5	5.33						

[1] Below.

[2] Above.

TABLE 15

Comparison of Milk Yield (Pounds) and Butterfat Percentage of Paternal Granddams and Dams, from the Records of the Jersey Registry of Merit

Item	Milk yield data		Butterfat percentage data	
	Granddam	Dam	Granddam	Dam
Average record	11,526±61	10,042±45	5.31±0.01	5.36±0.01
Standard deviation	3,336±43	2,491±32	.49± .01	.49± .01
Coefficient of variation	28.9	24.8	9.2	9.1
Correlation coefficient	0.20±0.02		0.06±0.02	

The average milk yield and butterfat percentage of these two groups, together with their variations and correlations are shown in table 15.

As indicated earlier, the milk yield of the paternal granddams is considerably larger than that of the rest of the breed. Their butterfat percentages are but average, however. The dams' production and butterfat percentage are comparable to those of the rest of the breed. The correlation coefficient for the milk yields of these two ancestors is 0.20 ± 0.02 and for the butterfat percentages 0.06 ± 0.03. Consequently the data show a little assortive mating for milk yield but none of any consequence for butterfat percentage. The correlations for these ancestors in the Jersey pedigrees are just a little higher than those for other breeds. The correlations for the paternal granddams and dams are: Holstein-Friesian, 0.14 ± 0.06 for milk yield and 0.001 ± 0.061 for butterfat percentage; and Guernsey, 0.13 ± 0.02 for milk yield and 0.01 ± 0.02 for butterfat percentage. The probable errors show that the values are comparable.

TABLE 16

Relation between the Paternal and Maternal Granddams' Milk Yield (Pounds) and Butterfat Percentages in 1,146 Pairs of Records

Paternal and maternal granddam, pairs (number)	Paternal granddam's milk yield	Maternal granddam's milk yield	Paternal and maternal granddam, pairs (number)	Paternal granddam's butterfat percentage	Maternal granddam's butterfat percentage
18	[1]7,000	8,280	21	[1]4.4	5.05
24	7,500	9,170	28	4.5	5.37
99	8,500	9,560	27	4.7	5.22
40	9,500	9,250	57	4.9	5.37
81	10,500	10,120	65	5.1	5.35
63	11,500	10,010	57	5.3	5.41
34	12,500	9,740	75	5.5	5.37
27	13,500	10,200	128	5.7	5.38
11	14,500	10,680	29	5.9	5.60
25	15,500	10,660	21	6.1	5.44
14	16,500	10,140	14	[2]6.2	5.36
75	17,500	9,850			
11	18,000	10,230			

[1] Below.

[2] Above.

Another measure of assortive mating as practiced in Jersey cattle breeding is the relation between the records of the paternal and maternal granddams. The average milk yields and butterfat percentages of the maternal granddams for given yields of the paternal granddams are seen in table 16. There are 1,146 pairs of paternal and maternal granddams in the pedigrees of these Jersey cattle. A slight trend is noted in the relation of the milk yields, but there is no particular change in the butterfat percentages of these grandparents. The data showing the variations and correlations of the two groups are given in table 17.

The selection of the sires on the basis of the record of their dams is again evident in these data; these cows producing 2,000 pounds of milk more than the dams of the cows to which the sires will be bred. These cows are not selected for their butterfat percentage. The standard deviation of the paternal granddams' milk yield has likewise markedly increased, the increase being correspondingly greater than the average milk yield, as shown by the larger coefficient of variation. The correlation coefficient for the milk yields of these granddams is statistically significant, showing that there is some assortive mating.

TABLE 17

Comparison of Milk Yield (Pounds) and Butterfat Percentage of Paternal and Maternal Granddams, from the Records of the Jersey Registry of Merit

Item	Milk-yield data		Butter at percentage data	
	Paternal granddam	Maternal granddam	Paternal granddam	Maternal granddam
Average record	11,847±102	9,807±70	5.34±0.01	5.37±0.01
Standard deviation	3,464± 72	2,366±49	.50± .01	.46± .01
Coefficient of variation	29.2	24.1	9.4	8.6
Correlation coefficient	0.12±0.03		0.13±0.03	

The degree of assortive mating in Jersey breeding as it is now practiced is essentially the same as that noted in the Holstein-Friesian and Guernsey breeds. The relations between the milk production and butterfat percentage of the dams and paternal granddams in these two breeds were found to be essentially like those given above, viz, 0.14 ± 0.06 and 0.13 ± 0.02 for milk yield and 0.00 ± 0.06 and 0.01 ± 0.02 for butterfat percentage. The similarity is also carried to the paternal and maternal granddam relations, the correlations being, for Holstein-Friesian, 0.30 ± 0.09 for milk yield and 0.04 ±

0.09 for the butterfat percentage, and Guernseys 0.10 ± 0.03 for milk yield and 0.12 ± 0.03 for butterfat percentage.

These assortive mating coefficients are all low. They are comparable to those recorded by Pearson and Lee (*18*) for stature, span, and forearm length in man. They are somewhat lower than those noted for weight, height at withers, depth at withers, heart girth, and paunch girth in a similar group of cows in this same Jersey breed, and they are much lower than those noted for the width at hips, body length, and rump length of Jersey cattle (*16*). The Jersey cattle, and quite possibly other breeds, are in the anomalous position of having the emphasis placed on their conformational points, the fancy points, rather than on the milk yield or butterfat percentage, the economic points in their breeding.

Relative Influence of Heredity and Environment on Milk Yield and Butterfat Percentage

The correlation coefficients for the different ancestral combinations furnish the material from which an estimate may be obtained of the relative importance of the effects of heredity and environment on milk production and butterfat percentage. Since the parents of a cow are reared to maturity some years before the animal's own growth period it is evident that the environment of the parent and offspring are likely to be quite different. Full and half sisters, on the other hand, are likely to be raised together at the same period of their development. Such being the case, the environmental influences of similar feeding, care, etc., would tend to make them alike. The effect of the similarity in conditions surrounding these sisters is represented in Wright's (*25*, *26*, *27*, *28*, *29*) terminology by e^2. The effect of heredity he calls h^2, and the determination of heredity by dominance deviation d^2, in the correlation between genotypes of different mates. The effects of the common environment and of dominance could theoretically be combined in all gradations. The analysis of the limiting cases (1) assuming dominance to be nonexistent, and (2) assuming the environmental effect to be absent is much simpler, however. By comparing the results of these analyses it is possible to determine the effect of each variable on the milk secretion and butterfat percentage of the Jersey cow. The correlations presented lead to the following equations:

Assortive mating is somatic and genetic factors are without dominance:

$$
\begin{aligned}
r_{sd} &= \frac{m}{h^2} & r_{so} = r_{do} &= 1/2h^2(1 + r_{sd}) \\
r_{oo} &= 1/2h^2(1 + m) + e^2 & r_{oo} &= 1/4h^2(1 + 2m + m^1) + e_1^2,
\end{aligned}
\tag{1}
$$

where s = sire, d = dam, o = daughter, oo = sister, and oo^1 = half sister.

Assortive mating as above but with genetic factors showing dominance and no effect of environment:

$$
\begin{aligned}
r_{sd} &= \frac{m}{h^2(1 - d^2)} & r_{so} = r_{do} &= 1/2h^2(1 - d^2)(1 + r_{sd}) \\
r_{oo} &= 1/2h^2\left(1 - \frac{d^2}{2}\right)(1 + m) & & r^1_{oo}1/4h^2(1 - d^2)(1 + 2m + m^1)
\end{aligned}
\tag{2}
$$

Assortive mating is genetic and no dominance of factors, environment as before:

$$
\begin{aligned}
r_{sd} &= h^2m & r_{so} = r_{do} &= 1/2h^2(1 + m) \\
r_{oo} &= 1/2h^2(1 + m) + e_1^2 & r^1_{oo} &= 1/4h^2(1 + 2m + m^1) + e_1^2
\end{aligned}
\tag{3}
$$

The numerical data necessary to complete this analysis as taken from the material previously presented are as follows: Correlation coefficients for the relation of the milk yields or butterfat percentages of half sisters with a common sire 0.24 and 0.25, sisters 0.39 and 0.41, half sisters with a common dam 0.19 and 0.2, daughter and dam 0.3 and 0.42. The assortive mating correlation between the sire and dam remains to be determined. The fact that the correlations between parent and offspring are 0.3 and 0.42, those between the paternal granddam and dam are 0.2 and 0.06, and those between the paternal and maternal granddams are 0.12 and 0.13, shows that the assortive mating correlations between the sire and dam are approximately 0.07 for milk yield and 0.01 for butterfat percentage. These correlations give the following values (table 18) for measuring the effect of dominance (d^2) and of variations in heredity (h^2) and environment (e^2), etc., on the Jersey cow's milk secretion and butterfat percentage.

The analyses of the data on the interrelation of the daughters', parents', and sisters' milk secretion allow us to form some estimate of the effects of inheritance, environment, and differences in dominance on the observed variation in milk yield and butterfat percentage. The analytical material tabulated presents the extreme hypotheses. Possibly these hypotheses could be modified and combined in all gradations between these extremes. The results from such combinations would be intermediate, however, so that conclusions reached on the basis of this material would be essentially correct for such intermediate hypotheses.

TABLE 18

Values for Measuring the Effect of Dominance and of Variations in Heredity and Environment, etc., on Milk Secretion and Butterfat Percentage of Jersey Cows

Item	h^2	e^2	m	m^1	d^2
Hypothesis (1):					
Milk yield	0.56	0.10	0.04	−0.21	....
Butterfat percentage	.83	−.01	.01	.09	....
Hypothesis (2):					
Milk yield	.96	....	.04	.49	0.41
Butterfat percentage	.79	....	.01	.04	−.05
Hypothesis (3):					
Milk yield	.53	.09	.13	−.28	....
Butterfat percentage	.83	−.01	.01	.08	....

The conclusions reached by hypothesis (1), assortive mating based on somatic resemblance and genetic factors without dominance, and (3), assortive mating genetic and no dominance of factors, are essentially the same, both qualitatively and quantitatively. Variations in heredity accounted for about half of the observed variations in milk yield and four-fifths of the observed variations in butterfat percentage. Environmental variations which have a common effect on the sisters' milk yield account for but 10 percent of the variation. On general grounds this slight effect of environment seems too little, but such may not be the case since the cows which are to make the Registry of Merit are apt to have a more uniformly good environment than possibly the majority of cows in the breed. The minus value of the correlation between the genotypes of the sire's mates appears to be contrary to what would be expected for the Jerseys since these mates would tend to be positively correlated on two grounds, namely, that they are apt to be related genetically, and that what selection is practiced is in the direction of making the cows similar in production. This contradiction is further emphasized by the correlation actually observed for the milk production ($r = +0.17$) and butterfat percentage ($r = 0.15$) of the sire's mates. A fairly large probable error is to be attached to the -0.28 observed, however, so that it may not be significant. Other than this discrepancy, the results of both analyses look quite reasonable.

Wide crosses of low and high milk-producing breeds have furnished definite evidence of the partial dominance of high milk production (*6*, *8*, *9*, *10*, *19*). If partial dominance does exist we should expect that hypothesis (2) would lead to a fair portrayal of the conditions in the breeding of these Jerseys. This analysis indicates that heredity determines 96 percent of the variation in milk yield and 79 percent of that in butterfat percentage. The assortive mating coefficients between the sire and cows to which he is bred are low for both the milk yield and butterfat percentage. The correlation for the sire's mates is positive and fairly large for milk yield, a fact that might be expected from a general knowledge of the Jersey breed. The same correlation for the butterfat percentage is low. These correlations, on the whole, agree better with those observed, 0.17 and 0.15, than in the two previous hypotheses. The effect of the dominance ratio is rather high. It does agree with the data on the crosses of breeds widely different in milk yield in showing more effect of dominance on milk yield than on butterfat percentage.

The probability seems to be that the hypothesis which would most nearly meet the conditions in these Jerseys is intermediate between those discussed. If this is so, it may be concluded that the inheritance of the cow controls between 50 and 70 percent of the variation in milk yield and between 75 and 85 percent of the variation in butterfat percentage. The effect of environment on milk yield is 5 to 10 percent. There is little relation between the sire's genotype and the genotypes of the cows to which he is bred, but there is a fair relation between the genotypes of these cows. Finally, the effect of dominance of genes for milk yield is fairly well marked, whereas that for genes for butterfat is small.

Heredity is even more prominent in the results from a similar analysis of the relations between the granddaughters, grandparents, and various cousin combinations. These analyses show that heredity accounts for 78 percent of the variation in milk yield and 95 percent of the variation in butterfat percentage. The effect of environment on the milk yield of the cousins was 13 percent, whereas that for the butterfat percentages was but 2 percent.

The genetic correlations between the genotypes according to hypothesis (1), assortive mating somatic without dominance, and hypothesis (3), assortive mating genetic without dominance, lead to much the same values. Determined on the basis of hypothesis (1), the parent and offspring correlations for milk yield and butterfat percentage are 0.54 and 0.51, respectively, the sister correlations are 0.52 and 0.50, respectively, and the half-sister correlations are 0.22 and 0.28. Determined on the basis of hypothesis (3), the same correlations are 0.56 and 0.51 for the parent and offspring and sisters and 0.25 and 0.28 for the half sisters. Again there is remarkable agreement between the results, either hypothesis apparently being adequate to explain them. The view consequently appears justified that the variation in the milk yields of these Jersey cows is largely due to heredity, environment playing but a small part. The theory that some of the factors for high milk yield show partial dominance also receives some slight support from this analysis. In the control of the variation of the butterfat percentage, heredity is the major factor, environment or dominance having little effect.

DISCUSSION

In the preceding section an attempt has been made to give definiteness to the terms "inheritance of milk yield" and "butterfat percentage" insofar as they relate to Jersey Registry of Merit cattle. The multiplicity of factors which have been shown to affect milk secretion have sometimes been allowed to cloud this issue. While the milk record of a cow is presumably the product of such independent characters as body constitution, good mammary-gland capacity, adequate secretory tissue, disease resistance, and breeding ability, and such environmental variables as adequate food for the full expression of the animals' inherent constitution for growth and production (*15, pp. 512–513*), so are most other inherited characters.

From the data in this paper it has been possible to separate roughly the variation of environment and the variation of the inheritance as they have influenced the milk secretion of these Jersey Registry of Merit cows. The analyses of these effects lead to the conclusion that the variations in feeding, care, and management of the cows, insofar as they are common to such close relatives as sisters, are much less important than those of heredity. The effect of the environment which is common only to the cow herself is more important than the environment common to sister cows. For milk yield the inheritance of the cow was found to control 50 to 70 percent of the variation in production; the environment common to sisters, 5 to 10 percent, and factors (some hereditary, such as dominance, and some environmental) common only to the cow herself, 20 to 45 percent. For the butterfat percentage the inheritance controls 75 to 85 percent of the variation, the environment common to sisters has no apparent effect, and the influences common only to the cow herself control 15 to 25 percent. Similar data obtained by the writer (*11*, *13*, *14*) on the Holstein-Friesian and Guernsey breeds led to similar conclusions.

The contribution which inheritance makes to the variation in the milk yields of these Jersey cows may itself be separated and the parts contributed by the various ancestors may be allotted to them. Individually considered, the immediate parents are found to contribute most, the effect of the sire's and dam's contribution to the daughter's milk secretion being essentially the same. No evidence that one parent consistently dominated the production of the offspring was found. The level of production which any cow has is the resultant of the interaction of the genes transmitted to it by its parents. The variation in the productions of the daughters of any pair of parents seems to be accounted for by the chance at segregation and fertilization of the factors contained in the daughter's sire and dam. The parents' genetic constitution thus has an average value for milk production as well as a prescribed range which it may transmit to its progeny, the factors transmitted by the male having an equal effect or "potency" with those transmitted by the female. This conclusion does not mean, as some have interpreted it, that the offspring of any given mating will have daughters whose milk yield will take a single value, a mean between the two parental productions; chance at maturation and segregation of the genes precludes that. The milk yield of the daughters, provided they are sufficient in number, will rather approach an average value determined by the genes possessed by the two parents, and will have a variation fixed by the segregation of their parents' heterozygous genes.

The correlation coefficients between the milk production of daughters and dams are 0.30 ± 0.01 for milk yield and 0.42 ± 0.01 for butterfat percentage. These coefficients are comparable to those for the Holstein-Friesian, 0.50 ± 0.02 for milk yield and 0.41 ± 0.02 for butterfat percentage, and for the Guernsey, 0.36 ± 0.02 and 0.42 ± 0.02. Similar daughter-dam correlations on butterfat yield have been noted by Turner (*23*) for Guernseys, 0.30, and for Jerseys (*24*), 0.35 ± 0.01; by Gifford as reported by Turner (*24*), for Ayrshires, 0.25 ± 0.02; Agar (*3*), for the Red Poll, 0.24 ± 0.06, and by Copeland (*4*) for Jerseys, 0.40 ± 0.03. Heizer (*17*), studying the records of a purebred Ayrshire herd, obtained daughter-dam correlations of 0.51 ± 0.03 for milk yield and 0.59 ± 0.02 for butterfat percentage. While certain of these correlation coefficients are statistically different from one another, their general trend points to a correlation between the daughter-dam milk yields or butterfat percentages of the different groups of dairy cattle of 0.4.

In a study of the daughter-dam correlations for eight different measurements of conformation in Jersey cattle (*15*), correlations of 0.37 were obtained between their estimated weights; 0.40 between their measured height at withers; 0.32 depth at withers; 0.42 heart girths; 0.35 paunch girths; 0.59 width at hips; 0.49 body length; and 0.64 rump length. These correlations, in contrast to those for milk yield, are influenced materially by a large assortive mating between the sizes of sire and dam. The data considering this assortive mating are comparable with those for milk yield and butterfat percentage as presented above.

Data quite similar to those contained in table 9 have been presented for Scottish Ayrshires (*20*, *21*, *22*). These data show the variance of the differences in production of the given pairs of ancestors rather than the correlation coefficients, a measure which leads to identical conclusions with the intraclass correlations. These variances for the butterfat percentage of granddaughters having A as the common grandparent are practically the same as those for granddaughters having C as the common grandparent. This fact is interpreted as showing that butterfat is autosomal in its inheritance. The earlier data on the butterfat percentage of the Holstein-Friesian and Guernsey breeds (*11, 13, 14*) have likewise been interpreted as leading to the same conclusion. The Jersey data in table 9 support this view. The variances in milk yields of these Ayrshires with A as the common grandparent are twice as large as those with C as the common grandparent. From this fact it is suggested that sex-linked factors for milk production may be present. There are, however, distinct contradictions to this interpretation within the data when taken as a whole. Data on the Holstein-Friesian cousins do lend some support to the sex-linked theory, although here, too, contradictions exist. The data in table 9 do not support the sex-linked hypothesis for the Jersey Registry of Merit cows. Similar data on the Guernsey breed likewise lend no support to it.

The data on the grandparents of these Jersey Registry of Merit cows show that, considered individually, the correlation between their milk yields and butterfat percentages and those of their granddaughters is only about half that of a parent production correlated with the production of the daughter. These grandparental correlations average about 0.2 while those of the parents average 0.4. In forecasting the record of a daughter, the record of a parent is four times as accurate as that of a grandparent, if we think of the degree of control as the square of their correlation coefficients. When the record used for forecasting a future cow's milk yield is that of a grandparent, the hazard evidently becomes much greater. This fact is emphasized in a recent paper by Edwards (*5*) in which the transmitting ability of a group of sires is compared. The production records of the dams of three sires in the same herd were given. The sire whose daughters had the largest production had the dam of lowest milk yield. His daughters' milk yield was 1,000 pounds higher than that of their dams, while that of sire 2's daughters was 4,350 pounds less than their dams, and that of sire 3's daughters was 3,510 pounds less. While this is a selected, and therefore striking case, not comparable with the average result, it is by no means unexpected in view of the grandparental correlations derived in this paper and in former papers as cited above. It seems to emphasize the fact that the difficulties surrounding the selection of a sire almost require that the constructive breeder raise and test his breeding bulls in his own herd.

The data in this paper demonstrate the inheritance of both the quantity of secretion which a gland manufactures and the proportion of a specific constituent of this secretion. An active gland may thus secrete a large volume of fluid, but this fluid may have a fairly wide variation in the percentage composition of its constituents. Similarly a gland of less active appearance may secrete a fluid which is highly concentrated, making its total secretion of the given constituent even greater than that of the apparently more active gland.

SUMMARY

This paper presents a study of the inheritance of milk yield and butterfat percentage in about 14,000 Jersey Registry of Merit cattle. The records of production are all of 364 to 366 days in length and are corrected to the production expected at maturity as judged by the cows' record obtained at another age. Parent and offspring, grandparent and offspring, sistership and cousinship correlations are presented. These correlations show that inheritance accounts for about half the observed variation in milk yield and four-fifths of the observed variation in butterfat percentage. The environmental variations which exist in these Jersey records account for little of the variation in the butterfat percentage and for only 10 percent of the variation in the milk yield. Dominance, assortive mating, and environmental variation common only to the cow herself account for the rest of the fluctuations in the yields and butterfat percentages.

LITERATURE CITED

(1) Anonymous.
1908. The probable error of a mean. By Student. Biometrika 6: 1–25, illus.

(2) ——
1915. On the distribution of the standard deviations of small samples: appendix I to paper by "Student" and R. A. Fisher. (Editorial) Biometrika 10: 522–529.

(3) Agar, W. E.
1926. Heredity and milking function. An analysis of the inheritance of milk and butterfat production in red poll cattle. Jour. Dept. Agr. Victoria 24: 1–9.

(4) Copeland, L.
1931. The contribution of the dam in inheritance of milk and butterfat. Jour. Dairy Sci. 14: 379–393, illus.

(5) Edwards, J.
1932. The progeny test as a method of evaluating the dairy sire. Jour. Agr. Sci. [England] 22: [811]–837, illus.

(6) Ellinger, T.
1923. The variation and inheritance of milk characters. Natl. Acad. Sci. Proc. 9: 111–116.

(7) Fisher, R. A.
1915. Frequency distribution of the values of the correlation coefficient in samples from an indefinitely large population. Biometrika 10: [507]–521.

(8) Gaines, W. L.
1923. The inheritance of fat content of milk in dairy cattle. Amer. Soc. Anim. Prod. Proc. 1922: 29–32, illus.

(9) Gowen, J. W.
1920. Inheritance in crosses of dairy and beef breeds of cattle. II. The transmission of milk yield to the first generation. Jour. Heredity 11: 300–316, illus.

(10) ——
1920. Inheritance in crosses of dairy and beef breeds of cattle. III. Transmission of butter-fat percentage to the first generation. Jour. Heredity 11: 365–376, illus.

(11) ——
1924. Inheritance of milk production. Sci. Agr. 4: 365–380, illus.

(12) ——
1924. Milk secretion; the study of the physiology and inheritance of milk yield and butter-fat percentage in dairy cattle. 363 pp., illus. Baltimore.

(13) ——
1927. Milk secretion as influenced by inheritance. Quart. Rev. Biol. 2: 516–531, illus.

(14) ——
1927. A résumé of cattle inheritance. Bibliographia Genetica 3: [87]–140.

(15) ——
1933. Conformation of the cow as related to milk secretion, Jersey registry of merit. Jour. Agr. Sci. [England] 23: [485]–513, illus.

(16) ——
1933. On the genetic constitution of Jersey cattle, as influenced by inheritance and environment. Genetics 18: 415–440.

(17) Heizer, E. E.
1933. The inheritance of milk production and butterfat percentage in a herd of pure-bred Ayrshire cattle. Amer. Soc. Anim. Prod. Proc. (1932) 25: 273–277.
(18) Pearson, K., and Lee, A.
1903. On the laws of inheritance in man. I. Inheritance of physical characters. Biometrika 2: 357–462.
(19) Russell, H. L., and Morrison, F. B.
1920. Inheritance of milk and meat production in cattle. Wis. Agr. Expt. Sta. Bull. 319: 53–54.
(20) Smith, A. D. Buchanan.
1928. On inbreeding in Jersey cattle. Brit. Assoc. Adv. Sci. Reprints (n.s.) 22: 649–655, illus.
(21) —— and Robison, O. J.
1931. The inheritance of milk yield. Vet. Rec. (n.s.) 11: 882–886.
(22) —— Scott, R. J., and Fowler, A. B.
1930. The inheritance of milk yield in Ayrshire cows. Jour. Dairy Research 1: [174]–179.
(23) Turner, C. W.
1925. A comparison of Guernsey sires based on the average "mature equivalent" fat production of the daughters and their dams. Mo. Agr. Expt. Sta. Research Bull. 79, 47 pp., illus.
(24) ——
1927. The mode of inheritance of yearly butterfat production; an analysis of the progeny performance of Jersey sires and dams. Mo. Agr. Expt. Sta. Research Bull. 112, 130 pp., illus.
(25) Wright, S.
1921. Systems of mating. I. The biometric relations between parent and offspring. Genetics 6: [111]–123, illus.
(26) ——
1921. Systems of mating. II. The effects of inbreeding on the genetic composition of a population. Genetics 6: [124]–143, illus.
(27) ——
1921. Systems of mating. III. Assortative mating based on somatic resemblance. Genetics 6: [144]–161, illus.
(28) ——
1921. Systems of mating. IV. The effects of selection. Genetics 6: [162]–166, illus.
(29) ——
1921. Systems of mating. V. General considerations. Genetics 6: [167]–178, illus.
(30) Young, A. W.
1916. Note on the standard deviation of samples of two or three. Biometrika 11: 277–280.

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INHERITANCE OF ABILITY TO LOCALIZE TOBACCO-MOSAIC VIRUS

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There have been many reports of inheritance of resistance to infection with virus diseases in plants. The following may be cited as examples. Allard (1, 2) noted that the hybrid *Nicotiana tabacum* L. × *glutinosa* L.[2] failed to show the mottling which is characteristic of *N. tabacum* when infected with tobacco mosaic, responding instead with necrosis like that of *N. glutinosa.* Smith (12) crossed mosaic-susceptible commercial varieties of spinach with a Manchurian spinach which escaped mosaic in the field, and found that resistance to infection was transmitted to the hybrid seedlings, and could be strengthened by selection among the hybrid strains. Lesley (8, 9) observed a tendency to escape curly-top infection in certain dwarf tomato varieties. He failed to transmit this tendency to standard, *i.e.*, non-dwarf, progenies after hybridization of resistant dwarfs with susceptible commercial varieties of standard habit, but recovered it in dwarf strains derived from hybrids. Bennett (3), in studying alpha-curl virus in the hybrid raspberry variety, Columbian, found recovery from disease symptoms, with disappearance of virus and renewal of susceptibility. It was believed that this hybrid was a seedling from the susceptible variety Cuthbert, pollinated by the immune variety Gregg. Mackie and Esau (10) reported resistance to curly top partially correlated with red color in bean hybrids. Porter (11) found hybrids between mosaic-resistant Chinese Long cucumbers and commercial susceptible strains to be susceptible, but less stunted than the susceptible parents.

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[2] *Nicotiana glutinosa* was referred to by Allard as *Nicotiana viscosum.*

The present paper describes a dominant Mendelian genetic factor that causes localization of tobacco-mosaic virus in inoculated leaves of garden pepper, *Capsicum frutescens* L., and a similar factor in each of certain Nicotiana L. and Solanum L. species. Plants possessing the hereditary factor for localization are susceptible to infection with tobacco-mosaic virus, but resistant to spread of virus in their tissues. It is believed that this is the first description of such a genetic factor affecting a virus disease in its plant hosts. The virus of streak disease of maize is influenced while in its insect host, the leafhopper *Cicadulina mbila* Naude, by a dominant, sex-linked, Mendelian factor, described by Storey (13). This dominant factor in the leafhopper confers ability to act as a vector of the disease. In a recent paper Storey (14) has thrown light on the nature of this hereditary character by demonstrating that the recessive-type, *i.e.*, inactive, insects may be made infective by wounding the wall of the intestine immediately before or after the insects are allowed to feed on diseased plant material, or by introducing virus directly into the insects' blood.

Capsicum Frutescens

Types of Response of Pepper Varieties to Infection with Typical Tobacco-Mosaic Virus

The majority of garden pepper varieties respond to infection with typical distorting-type tobacco-mosaic virus (5, p. 847) by mottling. In these varieties, yellowish primary lesions develop about 3 days after inoculation, and are followed by chlorosis of young leaves, with a prolonged period of reduced growth rate for the plant as a whole. Sometimes partial defoliation occurs. In time many mottled and distorted new leaves are formed. The yield of fruit of mottled plants is much less than that of healthy plants. As examples of varieties which give a mottling response, there may be cited the *Capsicum frutescens* varieties California Wonder, Celestial, Chinese Giant, Coral Gem Bouquet, Early Giant, Giant Crimson, Golden Dawn, Golden Queen, Hungarian, Large Bell, Oshkosh, Pimiento, Red Cherry, Red Japan Cluster, Ruby King, Spanish Monstrous, Upright Sweet Salad, and World Beater.

A few pepper varieties, on the other hand, respond by localized necrosis, without systemic mottling. Among the varieties tested, only two have consistently shown sharply-defined necrosis at the site of inoculation. These are *Capsicum frutescens* var. Tabasco, and *C. frutescens* var. *minimum* (previously grown under the name of *C. minimum* Blanco, but differing from common varieties of *C. frutescens* no more than these differ among themselves). These peppers are immune from the serious systemic effects of tobacco mosaic, although they are highly susceptible to infection. Many small necrotic lesions develop in inoculated leaves on the 2nd or 3rd day after inoculation. Virus may be recovered from these lesions, but not from green areas between lesions or from uninoculated parts of infected plants. Affected leaves drop off, usually between the 2nd and 5th days. Leaves with many lesions absciss earlier than those with few. The plants are free of virus as soon as all inoculated leaves have fallen. They are susceptible to repeated infection, but eliminate the virus promptly and grow essentially as normal plants.

Peppers giving these two kinds of response will be referred to as mottling-type and necrotic-type plants, respectively. For the purpose of studying the response of hybrids between mottling-type and necrotic-type plants, crosses were made between the mottling-type varieties Ruby King and Golden Dawn, and the two above-described necrotic-type varieties.

Response of Hybrids to Infection with Typical Tobacco-Mosaic Virus

Capsicum frutescens var. Tabasco × var. Ruby King.—Pollen from a plant of the mottling-type variety, Ruby King, was applied to stigmas of emasculated flowers of the necrotic-type variety, Tabasco. Many seeds were secured, but few germinated. Only 2 seedlings survived to blossom; both of these plants showed necrotic primary lesions after inoculation of leaves with typical distorting-type tobacco-mosaic virus. One of the 2 plants set no seed; the other bore many fruits, which were larger than those of the seed parent Tabasco, and when ripe partly pendant, like those of the pollen parent, Ruby King.

From seeds of the one original fertile plant, 48 second-generation seedlings were grown. Of these, 37 showed only necrotic primary lesions after inoculation with tobacco-mosaic virus, but 11 failed to show necrotic primary lesions and subsequently mottled. This reappearance of the mottling response in approximately a fourth of the second-generation hybrid plants suggested the possibility that a simple Mendelian dominant factor might be responsible for ability to localize the virus. The number of observed plants, however, was so small that this approach to a simple Mendelian ratio might have been accidental.

After the symptoms had been observed, the 48 infected plants were all set outdoors in a row in a garden. No attempt was made to prevent cross-pollination by insects. Eleven plants remained mottled and of high tobacco-mosaic virus content until autumn; the remaining 37, having lost their inoculated leaves, proved free of tobacco-mosaic virus. Some of the plants bore many fruits, others few or more. The 11 mottling-type plants bore comparatively few fruits, and, as later results showed, had probably received some pollen from nearby necrotic-type plants. Enough seeds were collected to grow seedlings from 36 necrotic-type plants and from 8 mottling-type plants.

Eleven of the 36 sets of seedlings from necrotic-type plants contained only necrotic-type plants, 1370 in all; the remaining 25 sets of seedlings from necrotic-type plants, a total of 2611, contained both necrotic-type and mottling-type individuals in each set in ratios approximating 3:1 (3.23:1 in the group as a whole). This seemed to indicate that 25 of the 36 necrotic-type F_2 plants had been heterozygous and 11 homozygous for a virus-localizing character. Combining these with the 11 plants that possessed the apparent recessive character, the ratios 11:25:11 were obtained, with one necrotic-type F_2 plant unclassified because of lack of seed. This is in good agreement with the expected 1:2:1 ratios characteristic of inheritance of a unit character in a second hybrid generation.

The 8 sets of seedlings from mottling plants did not contain mottling-type individuals only, as had been expected, but included some necrotic-type plants. The ratio was 50 necrotic-type to 208 mottling-type plants, or 0.24:1. This ratio is small, and it is believed that the necrotic-type plants occurring in these progenies were the result of natural cross-pollination from necrotic-type plants growing nearby. To eliminate such accidental cross-pollination, 36 mottling-type seedlings from a heterozygous necrotic-type seed-parent were grown in an isolated greenhouse. Seed was obtained from 20 of these, the others

setting no fruit. From this seed, 347 seedlings were grown. The seedlings were inoculated with tobacco-mosaic virus; none of them showed primary necrosis, but all developed mottling-type infections.

These results indicate beyond reasonable doubt that in *Capsicum frutescens* the ability to localize the virus of tobacco mosaic is transmitted in inheritance as a simple Mendelian dominant character, the lack of which allows movement of the virus to distant parts of inoculated plants, with resultant blanching, mottling, stunting, and reduction of crop, the symptoms commonly associated with the presence of the mottling type of tobacco-mosaic infection in peppers. The response of heterozygotes was indistinguishable from that of necrotic-type homozygotes. Lesions on inoculated cotyledons of necrotic-type plants resembled those on true leaves. The inoculation of variegated leaves, which occasionally appear on necrotic-type plants, produced approximately as many characteristic necrotic lesions in white, apparently chlorophyll-free, areas as in normal green areas.

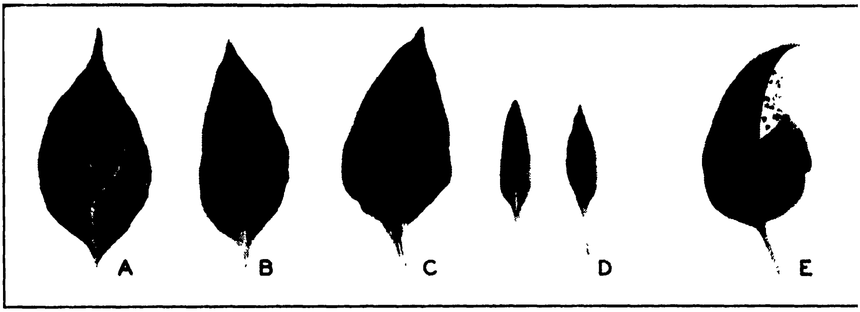

FIG. 1. Leaves of *Capsicum frutescens* inoculated with tobacco-mosaic virus. A. Necrotic primary lesions on leaf of a homozygous plant of constitution, *LL*. B. Lack of such lesions on a similarly treated leaf of a recessive individual, *ll*. C. Necrotic primary lesions on leaf of heterozygous individual, *Ll*. D. Similar necrotic primary lesions on cotyledons of necrotic-type plants. E. Similar lesions in both white and green areas of a variegated leaf.

Figure 1, (A), represents necrotic lesions on an inoculated leaf of a homozygous necrotic-type plant; (B), lack of such lesions on a similarly treated leaf of a mottling-type plant; (C), necrotic lesions on an inoculated leaf of a heterozygous individual; (D), necrotic primary

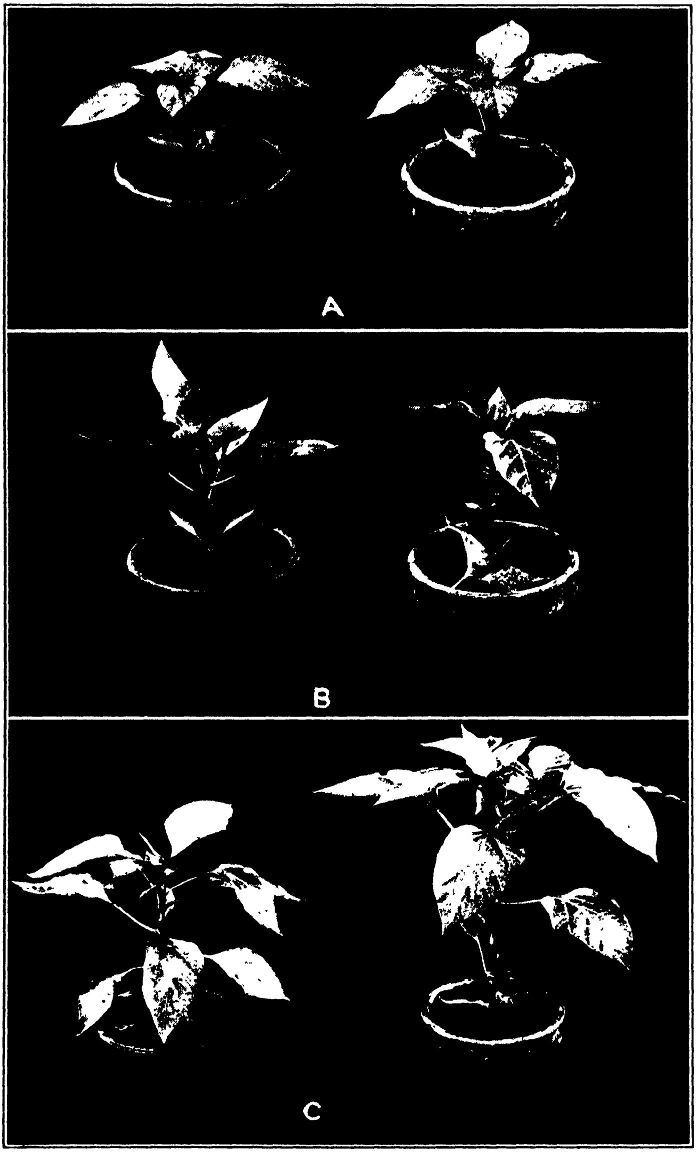

FIG. 2. Two plants of *Capsicum frutescens*, inoculated with tobacco-mosaic virus. The first was a mottling-type plant and the second, a necrotic-type. A. 3 days after inoculation of 2 leaves of each. B. 7 days after inoculation. Inoculated leaves had fallen from necrotic-type plant, freeing it from virus. C. 16 days after inoculation. Mottling-type plant was stunted and mottled. Necrotic-type one was large, without symptoms, and free of virus.

lesions on cotyledons; and (E), similar primary lesions in both white and green areas of a variegated leaf.

In figure 2 there are represented 2 plants, the first a mottling-type plant, the second a necrotic-type plant, (A) 3 days after inoculation of 2 leaves of each, (B) 7 days after inoculation, when the necrotic-type plant had lost its infected leaves, and (C) 16 days after inoculation, the mottling-type plant being then stunted and mottled, the necrotic-type plant larger, without symptoms of disease, and free of virus.

A fourth hybrid generation was grown, using in most cases seed from commercial, mottling-type *Capsicum frutescens* varieties, such as Ruby King, Golden Dawn, and California Wonder, fertilized with pollen from the third-generation, necrotic-type plants. The seed parents were thus homozygous for the absence of the character for localization, and the pollen parents possessed it either in the homozygous or the heterozygous condition. It was expected, therefore, that each set of seedlings would show either all necrotic-type, or one-half necrotic-type plants. This expectation was realized, for in 8 progenies there were altogether 201 necrotic type and no mottling-type plants, and in 7 progenies in which there were found both types, they were in approximately a 1:1 ratio, 94 seedlings proving to be of the necrotic type and 88 of the mottling type. The fourth generation thus added to the proof previously obtained, that the hereditary factor for localization of virus acts as a simple, dominant Mendelian factor. It also indicated that no incompatibility exists between the factor for localization of tobacco-mosaic virus and certain factors characterizing commercial types of peppers. The plants of this generation resembled commercial varieties in plant and fruit type more nearly than had the first 3 hybrid generations, yet expressed in full the characteristics of the necrotic-type response shown by Tabasco pepper and by plants of the earlier hybrid generations.[3]

Capsicum frutescens var. Golden Dawn × *var. minimum*.—A study was made of a second hybrid, *Capsicum frutescens* var. Golden Dawn

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[3] Subsequent back-crosses to commercial varieties have yielded individual plants bearing pendant, blunt-nosed, thick-walled, mild-flavored fruits as large as 95 x 50 mm., and reacting to leaf inoculation with tobacco-mosaic virus by formation of necrotic lesions, localization of virus, abscission of inoculated leaves, and subsequent healthy growth, just as in the original Tabasco pepper.

× var. *minimum*, in which more known genetic factors were concerned than had been the case with the Tabasco × Ruby King hybrid. *C. frutescens* var. Golden Dawn bears white flowers, green immature fruits, pendant, yellow, mild-flavored mature fruits with blunt apices, and is characterized by green cotyledons and hairy stem not branched at base. *C. frutescens* var. *minimum* has purple flowers, blackish-purple immature fruits, erect, red, pungent, mature fruits with pointed apices, purplish cotyledons, and smooth stem branching from base.

All plants of the first generation of *Capsicum frutescens* var. Golden Dawn × var. *minimum* showed purple-edged white flowers, immature fruits tinged with purple, partly pendent, red, pungent, mature fruits with pointed apices, green cotyledons, and nearly smooth stem branching at base. Twenty-six first-generation hybrid plants were grown. They were all inoculated with tobacco-mosaic virus, and all responded like the necrotic-type parent, showing necrotic primary lesions, abscission of inoculated leaves, and subsequent healthy growth.

In F_2 plants grown from seed of selfed F_1 plants an approximate 3:1 ratio between necrotic-type and mottling-type individuals was found, as expected; in a set of 1277 the ratio was 2.93:1. A group of 107 plants was examined for evidence of linkage between the factor for virus localization (*L*) or its recessive allelomorph (*l*) and several previously known genes[4] of the pepper: pungent (M) vs. mild (m) fruit, pendant (P) vs. erect (p) fruit, red (R) vs. yellow (r) mature fruit, purple (A) vs. white (a) flower, smooth (H) vs. hairy (h) stems, and pointed (D) vs. blunt-nosed (d) fruits. No evidence was found suggesting linkage between the gene for localization of tobacco-mosaic virus and any of the others. This is not surprising in view of the fact that there are 12 chromosomes in *Capsicum frutescens*.

Among 109 F_2 plants obtained as a result of the back-cross *Capsicum frutescens* var. Golden Dawn × (var. Golden Dawn × var. *minimum*), 54 proved to be of necrotic type and 55 of mottling type. No evidence was found for linkage between the gene for virus localization and any other.

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[4] The genes for purple flower and pendant, red, and pointed fruit are referred to in accordance with the usage of Deshpande, R. B., Studies in Indian chillies. (3) The inheritance of some characters in *Capsicum annuum* L. Indian Jour. Agr. Sci. **3**: 219–300. 1933.

Similar observations on a set of 113 plants derived by selfing a plant from the earlier Tabasco hybrid stocks gave no evidence of linkage between the factor for green (G) vs. pale (g) immature fruit and that for virus localization.

Data showing the distribution of the localizing gene and 7 other genes are summarized in table 1.

Response to Infection with Other Strains of Tobacco-Mosaic Virus

It was found possible to test the response of individual pepper plants to tobacco-mosaic virus without producing systemic infection even of mottling-type plants. This was done by picking a leaf from each plant to be tested, inserting its petiole into a test-tube full of water, and then inoculating the detached leaf. Leaves from necrotic-type plants responded with necrosis as they would if they were still attached to the plants. Leaves from mottling-type plants showed no necrosis, sometimes appearing unaffected by the inoculation, sometimes showing slight chlorosis in primary lesions, or, if yellowing from age, slight chlorophyll retention, as would be expected if the leaves were still attached to the plants.

TABLE 1

Absence of Linkage between the Virus-Localizing Factor (L) and Certain other Genetic Factors in Capsicum Frutescens

	Factors involved; L (localizing) and X	Observed ratios	Total number of observed plants
	X equals:	LX : Lx : lX : lx	
F_2 from selfed F_1; ratios expected in absence of linkage, 9:3:3:1	R (red)	49 : 22 : 26 : 9	106
	M (pungent)	55 : 15 : 21 : 14	105
	A (purple)	53 : 19 : 28 : 7	107
	P (pendant)	54 : 18 : 23 : 11	106
	H (smooth)	52 : 19 : 27 : 8	106
	D (pointed)	56 : 15 : 30 : 5	106
	G (green)	58 : 28 : 21 : 6	113
Back cross; ratios expected in absence of linkage, 1:1:1:1	R (red)	31 : 23 : 23 : 31	108
	M (pungent)	29 : 24 : 34 : 20	107
	A (purple)	27 : 27 : 33 : 22	109
	H (smooth)	27 : 26 : 33 : 22	108

This detached-leaf test allowed single plants to be tested for possible necrotic response to more than one strain of virus. Successive sets of leaves from plants of known response to the distorting strain of tobacco-mosaic virus were inoculated with the mottling and masked strains of tobacco-mosaic virus described elsewhere (5), and with virus of aucuba mosaic of tomato, propagated in *Nicotiana tabacum.* The set of 109 plants of *Capsicum frutescens* var. Golden Dawn × (var. Golden Dawn × var. *minimum*) already tested by the detached-leaf method for response to infection with the distorting strain of tobacco-mosaic virus, was used to supply leaves for tests with the other virus strains. Two of the plants of the set were not at the time available for this test, and among the 107 tested plants, leaves of which had been inoculated with the distorting strain of tobacco-mosaic virus, the ratio was 52 necrotic (heterozygous for localizing gene, *i.e.*, *Ll* plants) to 55 nonnecrotic (*ll* plants). The same 52 plants that responded with necrosis on leaves inoculated with the distorting strain of tobacco-mosaic virus also showed necrosis of the same sort on additional leaves inoculated with the two mild strains, the mottling and masked strains of tobacco-mosaic virus. The remaining 55 plants showed no necrosis after inoculation with these strains. Inoculation of a set of leaves with virus of aucuba mosaic of tomato in juice of Turkish tobacco plants induced typical necrotic primary lesions with pale brown center and dark brown periphery on the 52 necrotic-type plants of the set, but the formation of brown, imperfectly necrotic lesions on the leaves of all the mottling-type plants also.

When inoculated leaves were not detached from plants the response to these virus strains was as follows. Inoculation with the masked strain induced slight stunting and mild mottling in *ll* plants, necrosis and leaf abscission, with complete loss of virus in *LL* plants. Inoculation with the mottling strain induced somewhat similar stunting and mottling in *ll* plants, and the usual necrosis, abscission, and loss of virus in *LL* plants. Inoculation with the virus of aucuba mosaic of tomato induced severe stunting, blanching of young leaves, and occasional systemic necrosis in some *ll* plants, but imperfectly necrotic primary lesions, abscission of inoculated leaves, and loss of virus in others; all *LL* plants showed typical primary necrosis, leaf abscission, and loss of virus.

The similarity of necrotic primary lesions in all necrotic-type plants when infected with these 4 strains of tobacco-mosaic virus suggests that the factor that determines virus localization and necrotic response to infection with the common type of tobacco-mosaic virus may act similarly in response to infections with many, possibly all, strains of this virus.

Nicotiana

The simple Mendelian type of inheritance of the character for virus localization in Capsicum L. suggested the possibility that the same or a similar character might occur in other plants that show necrotic lesions when infected with tobacco-mosaic virus. Eight crosses were made between necrotic-type and mottling-type species and varieties of Nicotiana. In addition, 2 crosses between pairs of necrotic-type species and 1 cross between a pair of mottling-type species were tested. The number of plants involved was not large, but the response in hybrid progenies was definite, and tended to confirm the results obtained with Capsicum. Details of these experiments follow.

Nicotiana tabacum (mottling) × *glutinosa (necrotic)*. One Nicotiana cross between a mottling-type and a necrotic-type species was made by Allard (1) in 1914. This was the hybrid *N. tabacum* × *glutinosa*, the latter species being known to him not as *N. glutinosa*, but as *N. viscosum*. Allard found that the necrotic response of the pollen parent was transmitted to the first-generation hybrid. In view of the fact that the necrotic response of *N. glutinosa* had not been studied intensively at that time, it seemed desirable to obtain the hybrid again in order to determine whether the first-generation plants reproduced the necrotic response of the pollen parent fully, or in modified form.

The variety *purpurea* of the mottling-type species *Nicotiana tabacum* was chosen as seed parent and pollen from *N. glutinosa* was applied to it. The seeds thus obtained germinated well, and the seedlings which grew from them strongly resembled *N. tabacum* in leaf character and flower color. Inoculation of the leaves with tobacco-mosaic virus was followed, however, not by mottling, but by the appearance of necrotic spots closely resembling those produced in *N. glutinosa*. This response was given by all of 319 inoculated F_1 plants. To this extent the necrotic response appeared to be a dominant character, as was the case with the similar necrotic response in the pepper. Unlike the pepper hybrids, however, this Nicotiana hybrid was completely sterile, setting no seed during an entire year. For this reason the behavior of the factor responsible for the necrotic response has not been observed in a second or later generation. A photograph of the necrotic primary lesions in the first-generation hybrid is shown in figure 3, A.

Nicotiana bigelovii var. quadrivalvis (yellowing) × glutinosa (necrotic). —*N. bigelovii* Wats. var. *quadrivalvis* Pursh. is more severely affected than *N. tabacum* by infection with tobacco-mosaic virus. A prolonged period of stunting of the plant and yellowing of all young leaves precedes formation of mottled and distorted new leaves. The hybrid *N. bigelovii* var. *quadrivalvis* × *glutinosa* was tested to determine whether it would respond as had the hybrid *N. tabacum* × *glutinosa.* Necrotic primary lesions like those characteristic of *N. glutinosa* appeared on all of 135 inoculated plants of the first-generation hybrid. One hundred similar plants, not inoculated with virus, were grown through an entire summer. Their profuse blossoming did not result in seed formation. Because of this, no evidence was obtained for behavior of the factor for necrosis in later generations. It was evident, however, that in the first generation the necrotic response of *N. glutinosa* was fully dominant over the yellowing and mottling response of *N. bigelovii* var. *quadrivalvis,* as it had been over the mottling response of *N. tabacum* var. *purpurea.*

Nicotiana bigelovii var. quadrivalvis (yellowing) × sanderae (necrotic). —Similar evidence indicating dominance of the factor for necrosis in *N. sanderae* Sander was obtained from a single plant of *N. bigelovii* var. *quadrivalvis* (yellowing) × *sanderae* (necrotic), which was secured upon attempting to germinate seed formed as a result of the appropriate pollination. The hybrid bore large reddish-purple blossoms, but did not form seeds; its leaves resembled those of *N. bigelovii,* but were of coarser texture. Upon inoculation with tobacco-mosaic virus, it responded by formation of necrotic lesions resembling those of *N. sanderae* (Fig. 3, B).

Nicotiana caudigera (mottling) × acuminata (necrotic).—Although some Nicotiana hybrids formed no viable seeds, it was found possible to obtain several fertile hybrids, in which the responses of second-generation plants could be studied. One instance was that of the

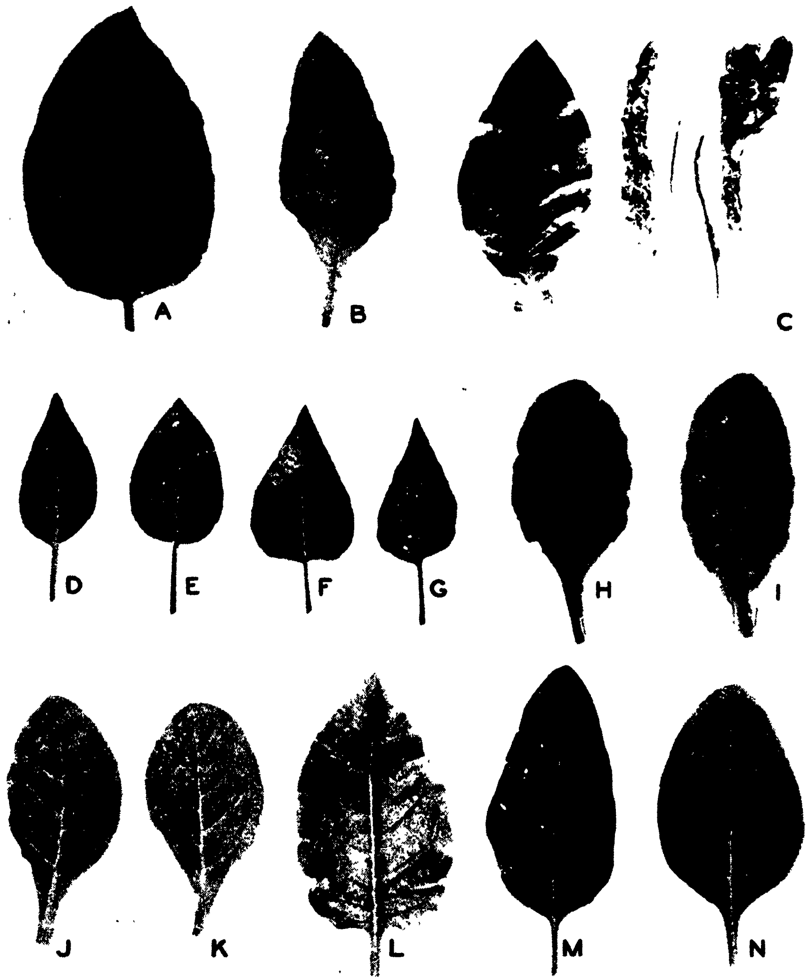

FIG. 3. A and B. Inoculated leaves A. *Nicotiana tabacum* var. *purpurea* × *glutinosa*. B. *N. bigelovii* var. *quadrivalvis* × *sanderae*. C. Healthy leaf and 4 systemically infected, mottled, and distorted leaves of *N. bigelovii* var. *quadrivalvis* × *tabacum* var. *purpurea*, showing enations. D–N. Inoculated leaves. D. *N. caudigera*. E. *N. acuminata* × *caudigera* F. *N. acuminata*. G. *N. caudigera* × *acuminata*. H. *N. sanderae* (mottling) × *langsdorffii*, recessive individual of F_2. I. *N. sanderae* (mottling) × *langsdorffii*, necrotic type individual of F_2. J. *N. langsdorffii* × *sanderae* (necrotic). K. *N. sanderae* (necrotic) × *langsdorffii*. L. *N. glutinosa* × *langsdorffii*. M. *N. paniculata* × *rustica* var. Winnebago. N. *N. glauca* × *langsdorffii*.

fertile hybrid *N. caudigera* Phil. × *acuminata* Hook. *N. caudigera* shows intense yellowing and stunting, followed by the production of distorted, mottled leaves after inoculation with tobacco-mosaic virus. *N. acuminata* shows no mottling, but only brown, necrotic primary lesions as a result of infection. The F_1 hybrid, *N. caudigera* × *acuminata*, showed necrosis like that of the pollen parent in all of 135 inoculated plants. The first-generation hybrid set seed abundantly. Seed from a single plant of this first generation was planted and germinated well. The seedlings were not uniform in appearance, some growing rapidly, others slowly. This was not unexpected, since *N. acuminata* had been observed to grow much more rapidly than *N. caudigera.* Altogether, 228 plants of the second hybrid generation were grown and inoculated with tobacco-mosaic virus; 193 responded with necrosis like that of *N. acuminata*, 35 with chlorosis like that of *N. caudigera.* The ratio of necrotic to chlorotic plants was therefore 5.5:1. The reciprocal F_1 hybrid, *N. acuminata* × *caudigera*, also proved to be of the necrotic type. Inoculated leaves of the two parent species and of reciprocal hybrids of the first generation are shown in figure 3, D to G.

The facts that the first-generation hybrids all showed a necrotic response, and that the second-generation plants were divided into two groups, giving respectively necrotic and chlorotic responses, seem to indicate that in Nicotiana, as in Capsicum, a dominant factor determines the necrotic response to infection with tobacco-mosaic virus, the corresponding recessive allowing systemic mottling. The reason for the excess of necrotic-type plants beyond the expected 3:1 ratio in the second generation is not known.

Nicotiana sanderae (mottling) × langsdorffii (necrotic). In commercial lots of seed of the ornamental, red-flowering *N. sanderae*, which originated many years ago from the cross *N. alata* Link & Otto × *forgetiana* Sand., the factor determining a necrotic response is typically present, but not invariably so. It is not unusual to obtain plants of *N. sanderae* that mottle when infected with tobacco-mosaic virus. A mottling-type plant of *N. sanderae* var. Crimson King was used as seed parent in crosses with *N. langsdorffii* Schrank, and *N. sanderae* (necrotic), both crosses producing fertile hybrids.

N. langsdorffii is not variable in response, as is *N. sanderae*, but responds invariably with necrosis. Only 13 first-generation hybrids were obtained of *N. sanderae* (mottling) × *langsdorffii.* Twelve of these responded to infection with tobacco-mosaic virus with necrosis similar to that shown by *N. langsdorffii.* The remaining plant responded with yellowing primary lesions, and later mottling, but showed evidence of being an actual hybrid by the form of its blossoms, which resembled those of *N. langsdorffii,* as did those of the other F_1 plants. Seed was obtained from the single mottling F_1 plant and from several of the necrotic-type F_1 plants.

A group of 57 plants from seeds of the one exceptional mottling F_1 plant showed typical localized necrosis in 10, which subsequently grew as healthy plants, mottling in 24, and systemic necrosis resulting in death in 23 individuals. The reason for failure of dominance of the necrotic response in the F_1 plant is not known. Some additional genetic factor may have operated in this exceptional case, preventing the necrotic response in the F_1 plant is spite of the presence of the factor for necrosis. The factor for necrosis was shown to have been present by its reappearance in the F_2 generation.

Seedlings derived from one of the 11 necrotic-type F_1 plants, upon inoculation with tobacco-mosaic virus, showed a necrotic-type response in 134 individuals, and a mottling-type response in the remaining 45, giving the ratio 2.98:1 (Fig. 3, H and I). The factor for necrosis in this instance appeared to be domiant in the usual sense.

Nicotiana sanderae (mottling) × *sanderae (necrotic).*—The same mottling-type plant of *N. sanderae* var. Crimson King used in the cross with *N. langsdorffii,* was used as seed parent in a cross with a necrotic-type *N. sanderae* plant. The F_1 hybrid, *N. sanderae* (mottling) × *sanderae* (necrotic) responded to inoculation with tobacco-mosaic virus by showing necrosis in all of 84 inoculated plants. In the second generation, 249 plants were inoculated; 185 responded with necrosis like that of the original pollen parent and of the F_1 plants, and 64 responded with chlorosis like that of the seed parent (ratio 2.88:1). The necrotic response in this cross appeared fully dominant, and no evidence of interference by other genetic factors was observed.

N. paniculata (mottling) × *rustica (necrotic).* Two other hybrids were produced between species showing necrosis and those not showing necrosis as a result of infection with tobacco-mosaic virus.

They were *N. paniculata L.* × *rustica* L. and *N. glauca* R. Grah. × *langsdorffii*; these were studied because of responses of the seed parents, one of which produces enations and the other of which is symptomless, or nearly so, when infected.

N. paniculata responds to infection with tobacco-mosaic virus by mottling, and by forming enations or tissue outgrowths (6). *N. rustica* responds with systemic necrosis when young, and by localized necrosis when older. Twenty-one plants of the first-generation hybrid, *N. paniculata* × *rustica* var. *jamaicensis*, all responded with necrosis closely resembling that of the pollen parent; the necrosis was systemic in young plants, but localized in inoculated leaves of older ones. *N. rustica* var. Winnebago shows necrotic lesions characterized by the formation of more dark brown pigment at the periphery of the lesion than occurs in the variety *jamaicensis*. Eleven plants of the hybrid *N. paniculata* × *rustica* var. Winnebago were inoculated, and 10 responded with lesions surrounded with dark brown pigment of the type of the pollen parent (Fig. 3, M). The 11th plant was atypical in appearance, being round-leaved and dwarfed; it also responded with necrosis, but not with dark brown pigment. The difference in type of necrotic lesion found in the two *N. rustica* varieties thus appeared, with the one exception noted, in the first generation hybrids. No sign of the tendency of *N. paniculata* to form enations appeared in any of the hybrids. Although only 32 F_1 plants were grown, they agreed with other crosses in producing all necrotic-type plants from mottling and necrotic-type parents.

Nicotiana glauca (nearly symptomless) × *langsdorffii (necrotic)*. *N. glauca* mottles faintly if infected with tobacco-mosaic virus when young. In older plants the virus multiplies well in inoculated leaves, moves toward the top of the plant erratically, and when present in the younger leaves sometimes causes traces of mottling, and sometimes no symptoms. *N. langsdorffii*, as stated before, shows a necrotic response with production of black-brown pigment at the periphery of primary lesions. A set of plants of the first generation of *N. glauca* × *langsdorffii* was grown and 198 plants were inoculated; all responded with necrosis (Fig. 3, N) resembling that characteristic of *N. langsdorffii*.

Nicotiana langsdorffii (necrotic) × *sanderae (necrotic)*. If the apparently similar genetic factors for necrosis were fundamentally unlike in some of the species already mentioned, combining such dominant factors in a single F_1 plant might result in a modified response. Only two pairs of necrotic-type species were tested. The first of these was *N. langsdorffii* × *sanderae* (necrotic). The response of the first-generation hybrid did not differ from that of the parent species; necrotic lesions surrounded by zones of blackish brown pigment (Fig. 3, J) were produced in all of 162 F_1 plants. There appeared to be no additive effect upon combining dominant factors from the two parents in individual hybrid plants. The reciprocal hybrid, *N. sanderae* (necrotic) × *langsdorffii* (Fig. 3, K), was also grown and 92 plants, after being inoculated with tobacco-mosaic virus, all showed a necrotic response like that of the parents and of *N. langsdorffii* × *sanderae.* If the genetic factors in the two parents were similar in nature, but not located in corresponding chromosomes, some plants of a second generation should fail to receive the factor for necrotic response from either parent, since there would be independent segregation. In a second generation grown from seeds of selfed *N. sanderae* × *langsdorffii* plants, all of 255 inoculated plants showed necrotic primary lesions of the type shown in the first hybrid generation. The dominant genetic factors determining the necrotic response in these two Nicotiana species acted as though identical, giving no obvious additive effect when combined, and appearing in all F_2 plants.

Nicotiana glutinosa (necrotic) × *langsdorffii (necrotic).* The second combination of two necrotic-type species was that of *N. glutinosa* and *N. langsdorffii.* Only a single plant of the hybrid *N. glutinosa* × *langsdorffii* grew from seed obtained as a result of appropriate pollination. It was intermediate in appearance between the unlike parent species and proved self-sterile and sterile to *N. langsdorffii* pollen. Upon inoculation with tobacco-mosaic virus it formed necrotic primary lesions (Fig. 3, L) closely resembling those of *N. glutinosa*, but surrounded by a somewhat darker brown peripheral zone, only a little less blackish than that characteristic of *N. langsdorffii.*

No evidence was found in the behavior of the two necrotic-type combinations, *N. sanderae* (necrotic) × *langsdorffii* and *N. glutinosa* × *langsdorffii*, to indicate that the dominant factors determining necrotic response are different in the species *N. sanderae*, *N. langsdorffii*, and *N. glutinosa.*

Nicotiana bigelovii var. quadrivalvis (yellowing) × *tabacum (mottling).* —Since the hybrids of *N. tabacum* and *N. quadrivalvis* with *N. glutinosa* had resulted in F_1 plants of necrotic type, it was desirable to test the hybrid *N. bigelovii* var. *quadrivalvis* × *tabacum*, although it seemed unlikely that there would be production of necrotic-type plants without the use of a necrotic-type parent. The first-generation hybrid *N. bigelovii* var. *quadrivalvis* × *tabacum* var. *purpurea* was produced and about 100 plants were grown to blossoming, sets of the plants being inoculated at intervals with tobacco-mosaic virus.

TABLE 2

Summary of Tests Showing Behavior of Factor for Necrosis in Nicotiana Hybrids

	First hybrid generation		Second hybrid generation		
	Necrotic-type plants	Mottling-type plants	Necrotic-type plants	Mottling-type plants	Ratios
Nicotiana tabacum × *glutinosa*	319	0	...	...	.....
Nicotiana bigelovii var. *quadrivalvis* × *glutinosa*	135	0	...	...	.....
Nicotiana bigelovii var. *quadrivalvis* × *sanderae* (necrotic)	1	0	...	...	.....
Nicotiana caudigera × *acuminata*	135	0	193	35	5.5:1
Nicotiana sanderae (mottling) × *langsdorffii*	12	1	134	45	3.0:1
Nicotiana sanderae (mottling) × *sanderae* (necrotic)	84	0	185	64	2.9:1
Nicotiana paniculata × *rustica*	32	0	...	...	.....
Nicotiana glauca × *langsdorffii*	198	0	...	...	.....
Totals	916	1	512	144	3.6:1

The hybrid stock responded to infection by stunting, distortion, and mottling. No necrosis appeared at any time. Outgrowths of tissue (enations) from the lower surface of mottled leaves were numerous in the infected plants (Fig. 3, C), although these had been grown in shade, a condition not suitable for formation of enations on plants of *N. paniculata*, as shown by Jensen (6).

Neither the failure to secure plants of a second generation from some Nicotiana hybrids, nor the small numbers of F_1 plants obtained from certain crosses need obscure the result of the study of this genus as a whole. No deviation from the parental responses was found when two necrotic-type species were crossed with each other. A summary of tested plants in eight crosses between mottling-type and necrotic-type plants is shown in table 2. Altogether, 917 F_1 plants were inoculated with tobacco-mosaic virus; all but one individual responded with necrosis. The single exception had as one parent the rather variable horticultural form known as *N. sanderae*; some interfering genetic character or combination of characters may have been responsible for the anomalous result. From 3 of the 8 crosses a second generation was secured by selfing plants of the F_1 generation. A total of 656 F_2 plants were inoculated. A considerable excess of necrotic-type plants occurred in the F_2 generation of *N. caudigera* × *acuminata* (193 necrotic-type; 35 mottling-type); the other F_2 sets gave ratios of 3.0:1 and 2.9:1 respectively. The conclusion seems warranted that in the genus Nicotiana, as in Capsicum, a necrotic response to infection with tobacco-mosaic virus acts as a typically dominant character in certain crosses between necrotic-type and mottling-type forms.

Solanum Melongena

In the genera Capsicum and Nicotiana, factors for necrotic response to inoculation with tobacco-mosaic virus behaved similarly, being in both cases dominant over factors for mottling response. In the genus Solanum, the species *S. melongena* L. (eggplant) shows both necrotic and mottling types of response among its varieties. It seemed probable that in this genus also the factor for necrosis might be dominant over that for mottling. This was not found to be entirely true, however.

Solanum melongena var. Peking Green mottles inconspicuously if infected with tobacco-mosaic virus when young; infected plants tend to become symptomless with age. The variety Hangchow Long is symptomless regardless of age at time of inoculation, but allows virus to increase in its tissues and to spread systemically. The variety Black Beauty is killed by systemic necrosis if infected as a small seedling, but localizes the virus completely and shows necrosis only at the site of inoculation on leaves of older plants. The mottling-type variety Peking Green and the symptomless variety Hangchow Long were both pollinated from the necrotic-type variety Black Beauty.

Solanum melongena var. Peking Green × var. Black Beauty.—The first-generation hybrid *S. melongena* var. Peking Green × var. Black Beauty responded to infection with tobacco-mosaic virus in all of 103 inoculated plants by production of faintly yellowish primary lesions. Around most of these lesions, however, flecks of necrosis later appeared, sometimes developing as thin, light brown, necrotic, circular or wavy lines. A faint systemic mottling later developed, again marked by occasional thin, light brown, necrotic concentric rings and other necrotic markings outlining infected areas. The leaves affected by the systemic spread of the virus were characterized by not being so flat as the corresponding leaves of healthy plants; cup-like depressions and slightly rugose leaf surface seemed to be caused by irregularities in rate of expansion of the lamina. The first-generation hybrids tended toward a symptomless condition in later growth. It was evident that the F_1 plants did not respond like either parent.

A second hybrid generation was grown from seed of several plants of the first-generation hybrid. Among the second-generation plants the solidly necrotic, dark brown primary lesions and similar secondary lesions in young plants characteristic of the necrotic-type parent, Black Beauty eggplant, reappeared in some plants. A considerable part of the remaining individuals showed flecks of ring-necrosis such as had characterized infected F_1 plants. It seemed probable that in this Solanum hybrid the necrotic response was not wholly dominant, although a single pair of allelomorphs might possibly account for the responses. The heterozygous plants were irregular in time of formation of traces of necrosis. For this reason attention was directed to observing the ratio of fully necrotic F_2 plants to those showing traces of necrosis or none at all. This ratio, 132:333, amounted to about 1:2.5, and so might be considered to support to some extent the hypothesis that a single Mendelian factor for necrosis existed in *Solanum melongena*, as in *Capsicum frutescens*, and some Nicotiana species, although in the case of *S. melongena* dominance appeared imperfect, and the heterozygotes could not be distingushed readily from the non-necrotic segregates.

Solanum melongena var. Hangchow Long × var. Black Beauty.—The first-generation hybrid *S. melongena* var. Hangchow Long × var. Black Beauty responded to infection with tobacco-mosaic virus in all of 150 inoculated plants by production of yellowish primary lesions peripherally marked with traces of necrosis and by necrotic flecks on younger leaves. The symptoms were similar to those on the F_1 Peking Green × Black Beauty eggplant. In the second generation, a fully necrotic response, like that of Black Beauty eggplant, was noted in 199 of 743 inoculated plants. This gives the ratio of fully-necrotic plants to those not fully necrotic or showing no necrosis as about 1:2.7, a slightly closer approach to a 1:3 ratio than had been obtained with the Peking Green × Black Beauty hybrids.

This second *Solanum melongena* hybrid confirms the conclusion reached from observation of the preceding form, that the necrotic response in this species is not fully dominant, but is probably of the nature of a Mendelian character, as in Nicotiana and Capsicum species.

DISCUSSION

The characteristic of localizing tobacco-mosaic virus, because of its easy manipulation as an hereditary unit, may be a useful tool in attempting to gain an understanding of the nature of viruses. A plant that possesses the dominant character is able to restrain tobacco-mosaic virus from producing a systemic infection, although without this factor it would be unable to do so. It has been shown in another paper (5) that several distinct strains of tobacco-mosaic virus differ in degree of freedom to spread in systemic infection of young leaves. In an earlier paper (4, p. 341) it was shown that a similar situation was discernible in iodine-stained material of various hosts of the distorting strain of tobacco-mosaic virus. It has been shown by Kunkel (7) that the virus of aucuba mosaic is in many characteristics similar to that of tobacco mosaic, but it is restricted to necrotic primary lesions in *N. sylvestris* Spegaz. & Comes, in which tobacco-mosaic virus produces a systemic mottling. It appears that slight differences in strains of host plants, typified by the difference of a single genetic factor in the pepper, and slight differences in strains of virus which are in most respects similar, may both make differences in freedom of virus spread and even the difference between a localized necrotic infection and one of systemic mottling. Physical or chemical characteristics of the virus, as well as of the host plant, are probably concerned in this relationship.

SUMMARY

Localization of tobacco-mosaic virus in *Capsicum frutescens*, the garden pepper, was found to be determined by a dominant Mendelian factor. In plants possessing this factor the virus increased in tissues at the site of inoculation and caused the appearance of numerous necrotic spots of small size, with early abscission of the inoculated leaf and subsequent healthy growth of the plant to maturity. The recessive allelomorph allowed systemic spread of virus, stunting of plant, mottling and distortion of leaves, and reduced yield of fruit.

Somewhat similar genetic factors, determining necrotic response, were found in a number of Nicotiana species and in *Solanum melongena*.

LITERATURE CITED

1. Allard, H. A. The mosaic disease of tobacco. U. S. Dept. Agr. Bull. 40. 1914.
2. ———. A specific mosaic disease in *Nicotiana viscosum* distinct from the mosaic disease of tobacco. Jour. Agr. Res. **7**: 481–486. 1916.
3. Bennett, C. W. Further observations and experiments on the curl disease of raspberries. Phytopath. **20**: 787–802. 1930.
4. Holmes, F. O. Symptoms of tobacco mosaic disease. Contrib. Boyce Thompson Inst. **4**: 323–357. 1932.
5. ———. A masked strain of tobacco-mosaic virus. Phytopath. **24**: 845–873. 1934.
6. Jensen, J. H. Leaf enations resulting from tobacco mosaic infection in certain species of Nicotiana L. Contrib. Boyce Thompson Inst. **5**: 129–142. 1933.
7. Kunkel, L. O. Studies on acquired immunity with tobacco and aucuba mosaics. Phytopath. **24**: 437–466. 1934.
8. Lesley, J. W. A study of resistance to western yellow blight of tomato varieties. Hilgardia **2**: 47–66. 1926.
9. ———. The resistance of varieties and new dwarf races of tomato to curly top (western yellow blight or yellows). Hilgardia **6**: 27–44. 1931.
10. Mackie, W. W., and K. Esau. A preliminary report on resistance to curly top of sugar beet in bean hybrids and varieties. Phytopath. **22**: 207–216. 1932.

11. Porter, R. H. The resistance of cucumbers to mosaic. Phytopath. **20:** 114. 1930. (Abst.)
12. Smith, L. B. Breeding mosaic resistant spinach and notes on malnutrition. Virginia Truck Exp. Sta. Bull. 31–32: 137–160. 1920.
13. Storey, H. H. The inheritance by an insect vector of the ability to transmit a plant virus. Proc. Roy. Soc., Ser. B. **112:** 46–60. 1932.
14. ———. Investigations of the mechanism of the transmission of plant viruses by insect vectors.—I. Proc. Roy. Soc., Ser. B. **113:** 463–485. 1933.

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MULTIPLICATION OF THE VIRUSES OF TOBACCO AND AUCUBA MOSAICS IN GROWING EXCISED TOMATO ROOT TIPS

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Stock supplies of plant-disease viruses are at present maintained in several different ways. One common method is to preserve samples of filtered juice from infected plants in a frozen state in a refrigerator. This method is fairly satisfactory for the maintenance of viruses that retain their activity for reasonably long periods of time under such conditions. It is not satisfactory for all viruses, however, since many soon become inactivated, even when frozen.

Another standard method of maintaining stocks is by inoculation of plants kept in greenhouses or in insect-proof cages. Pieces of tissue from such infected plants can be used at any time as a source of inoculum. The virus, under these conditions, is maintained in a state of active multiplication. Greenhouse or caged plants, however, require much space and special attention. Moreover, with plants grown in greenhouses or cages, the possibility of accidental contamination by insect, human, or other agencies is always present. The purity of strains so maintained can, therefore, not be absolutely relied upon. Attempts have been made to grow host plants under isolated, aseptic conditions free from this danger, but the techniques so far developed are rather cumbersome. No simple reliable method of maintaining stocks of plant-disease viruses under conditions of known purity, free from danger of contamination, and in a state of active multiplication, has been developed to date. Such a method would obviously be of value, especially where many different viruses are being studied in the same laboratory.

The active principle of several plant-virus diseases is known to be present in the roots of plants systemically infected with these diseases and to increase in concentration with progress of time after infection. Since it has been shown that root tips of the tomato, a plant that is susceptible to many different virus diseases, are capable of growing apparently indefinitely in an isolated state under controlled environmental conditions (32), it has seemed possible that such roots might prove to be excellent experimental material in which to maintain certain viruses under controlled conditions. Such cultivation of the viruses would depend (1) on the infected roots' growing sufficiently well to maintain cultures, and (2) on the multiplication of virus in the roots. No marked symptoms have been observed in roots of diseased plants in spite of the presence and increase of virus in such roots. For this reason it has sometimes been supposed that multiplication of virus does not take place in roots, but that its presence and increase in roots may be due to accumulation of virus produced elsewhere in the plants. The work presented here was planned to test some of these possibilities.

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EXPERIMENTAL

The stem of a rapidly growing plant of Bonny Best tomato carrying a pronounced systemic infection with tobacco-mosaic disease was cut up into segments. These were thoroughly washed and so suspended, by single threads in 3-liter Erlenmeyer flasks containing a little water, that the cuttings did not touch either the flask or the water. The flasks were plugged with cotton and left on a laboratory table. Roots developed on the basal portions of the cuttings. After 11 days, 18 root tips were carefully removed and placed in 125-ml. Erlenmeyer flasks, prepared as previously described (31). Each flask contained 50 ml. of nutrient. The nutrient used was prepared as previously described (32) and contained the following substances in the concentrations indicated:

$Ca(NO_3)_2$	0.60 millimols
$MgSO_4$	0.30 "
KNO_3	0.80 "
KCl	0.87 "
KH_2PO_4	0.09 "
$Fe_2(SO_4)_3$	0.006 "
Sucrose	2% (by weight)
Yeast (extract)	0.01%

Of the 18 roots cultured, 7 became infected with bacteria and had to be discarded, 1 failed to grow, 2 made poor growth and were discarded, 6 were accidentally infected with fungi, and 2 remained in good condition. Of the 6 contaminated roots, 2 succumbed, while 4 were recovered in an aseptic condition by cutting away and discarding the contaminated portions. There were thus 6 roots from which to build up a diseased clone. At the end of a week the 6 surviving cultures were cut into pieces approximately 10 mm. long; the apices were transferred to fresh flasks together with such subapical pieces as were desired for additional cultures, and the discarded basal portions were tested for presence of virus by crushing and rubbing into leaves of *Nicotiana glutinosa* L. All 6 roots contained virus, the individual roots producing from 22 to about 500 lesions. A single root tip was selected for further study. It served as the parent stock from which all subsequent cultures were derived.

The clone was allowed to grow under controlled laboratory conditions and was subcultured (32) at weekly intervals for a period of 30 weeks. At the 4th passage it contained 50 cultures. This number was maintained through passages 4, 5, 6, and 7, after which enough roots were discarded to reduce the number to 25. The latter number was maintained through all subsequent passages.

At the end of each of the first 4 passages, each culture was tested for presence of virus by inoculation of plants of *Nicotiana glutinosa.* In all passages thereafter, with the exception of the 20th and 30th, only every 5th culture was tested. The virus was present in each root tested in every passage, and although the titer, as shown by the mean number of lesions obtained per culture, varied quite widely from passage to passage, the observed variations were in general apparently related to differences in host-tissue growth rates brought about by external factors. This relationship is shown in figure 1.

At the end of the 20th and the 30th passages, inoculations were made from cultures Nos. 5, 10, 15, 20, and 25 into plants of *Nicotiana tabacum* L. and *N. sylvestris* Spegaz. and Comes to determine whether the virus was still capable of producing typical symptoms in these plants. At the same time, tissue from each root was inoculated into a leaf of *N. glutinosa* to determine whether virus was present in every culture. At the end of 20 passages, inoculum prepared from every culture except No. 25 induced characteristic local lesions on

N. glutinosa. Failure in this case was probably due to accidental failure to inoculate the test plant, since the infections produced by other pieces of the same root on plants of *N. tabacum* and *N. sylvestris* were fully systemic and showed no noticeable abnormalities. The results obtained by tests made at the end of 30 passages confirmed those secured by inoculations from roots in the 20th passage. Thirty passages in growing isolated root tips had thus apparently left the virus unchanged in behavior. The theoretical multiplication of host tissue during 30 weeks is estimated to have been of the order 10^{23} (32). Since the virus showed no consistent detectable diminution in titer in

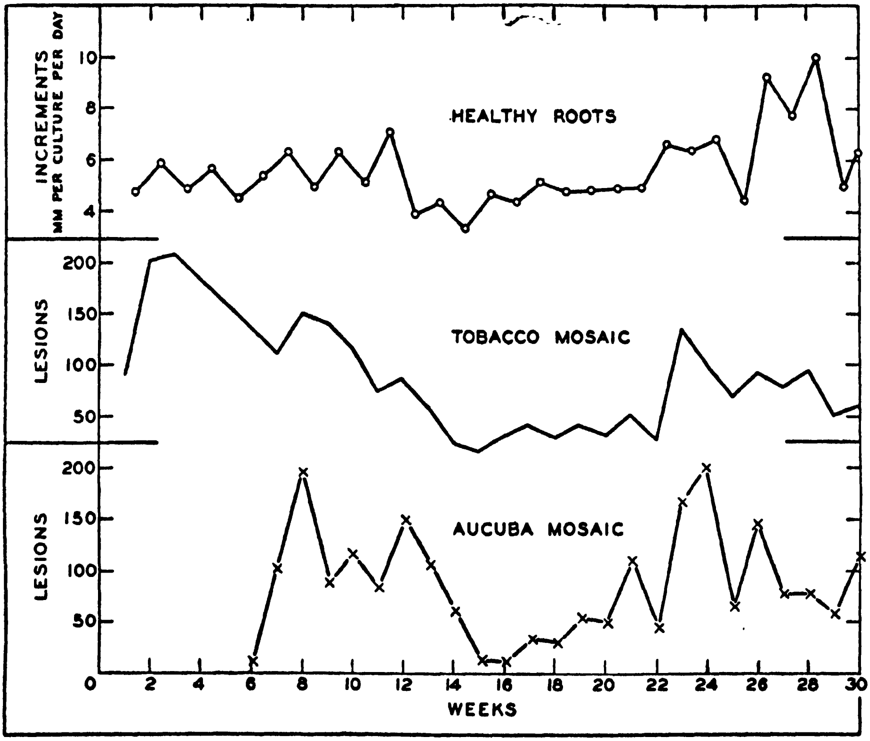

FIG. 1. Weekly variations in titer of tobacco-mosaic virus and aucuba-mosaic virus multiplied in growing isolated root tips and the simultaneous growth rates of healthy roots. The correspondence between the low increment rates from the 12th to the 22nd weeks and the low virus titer obtained in these same weeks is evident.

this time, it is assumed that its increase had kept pace with the multiplication of the host tissue. The virus is being maintained in further subcultures.

Aucuba mosaic was cultivated in the same way through 25 weekly passages in root tips derived from a single cutting of a systemically infected plant. Virus was recovered from these roots by inoculation into *Nicotiana glutinosa* at the end of every passage. At the end of 20 passages, all roots were tested for the presence of virus by inoculation into *N. glutinosa*, and Nos. 5, 10, 15, 20, and 25 were tested for the characteristic symptoms on *N. tabacum* and *N. sylvestris*. The disease produced in these plants was typical in every instance. The infection on *N. sylvestris* showed the characteristic necrotic local lesions without becoming systemic, while on *N. tabacum* it was systemic and characterized by the usual mottling symptoms. The virus had apparently been unaltered by cultivation in isolated tissues through 20 passages, and, although not subsequently tested for symptom expression, was shown to be present after 25 passages. It is being maintained in further subcultures.

Roots infected with each of these viruses were carefully examined for visible symptoms of disease. They were found to be as straight and clean, with as many and as uniform branches, as were the healthy ones. No macroscopic lesions, discolorations, distortion of tissue, change in manner of growth, or other symptoms of disease, could be observed. It is possible that a detailed histological study may disclose the existence of symptoms not visible macroscopically, but it is a remarkable fact that agents that produce such marked distortion, discoloration, and stunting in the aerial parts of the plant as do these two viruses, should leave the roots, even when carrying large amounts of virus, apparently unaffected. Although growth rates are an unreliable criterion for comparison, unless the different cultures to be compared are all derived from the same clonal parent, it is noteworthy that cultures of the clones carrying these viruses grew as rapidly and appeared in as good condition as did those of healthy clones that were being maintained during the time these experiments were in progress.

Experiments were carried out to determine if virus grown in isolated roots escapes into the surrounding medium or can infect healthy roots when added to the medium and whether healthy roots become infected when wounded in the presence of virus or when grown in the same flasks with and in contact with diseased roots. Four rapidly growing cultures infected with tobacco-mosaic disease were selected at the end of the 3rd passage, and a portion of the nutrient from each flask was tested for presence of virus by rubbing into leaves of *Nicotiana glutinosa.* No lesions were produced by the nutrient from any of the 4, while the roots grown in these flasks produced 39, 40, 28, and 253 lesions, respectively. Ten healthy roots, grown in media to which virus was added, did not become infected. In another group of experiments, 12 rapidly growing roots infected with tobacco-mosaic disease, in the 9th passage, were split lengthwise for about 2 mm. Pieces of healthy roots in the 36th passage (clone C, see 32) were then "threaded" through these diseased pieces. Both diseased and healthy fragments grew actively and at the end of 12 days the pieces were removed and tested separately for presence of virus. The originally healthy fragments in no case produced any lesions, while the originally diseased roots produced from 50 to 200 lesions each (average 114 lesions) on comparable test plants. In still another experiment, roots in the 6th passage carrying tobacco-mosaic virus were placed in the bottom of a Petri dish and crushed with a glass spatula. Ten roots of a healthy clone in the 32nd passage were laid in the juice of these diseased tissues and repeatedly wounded with fine insect pins. They were then removed to fresh nutrient and allowed to grow for one week and subcultured and the discarded portions tested for virus. Five of the 10 roots survived and, when tested, produced a few (1 to 47) lesions on leaves of *Nicotiana glutinosa.* At the end of the 2nd passage after inoculation, however, all of the 16 cultures obtained from the 5 surviving roots were virus-free. In a second series of cultures, 10 roots inoculated in the same way were likewise all virus-free in the second passage. Diseased clones of roots have not been established by direct inoculation. They have only been obtained by growing roots from systemically infected plants. Although the experiments performed are insufficient to warrant the conclusion that roots cannot be infected in these ways, it at least appears that the virus does not ordinarily escape from the diseased tissues into the medium, nor is the disease easily transferred to healthy roots by contact with diseased roots or with media to which virus has been added. Isolated roots are evidently refractory material as far as inoculation by these methods is concerned.

DISCUSSION

It has been shown that the viruses of aucuba mosaic and tobacco mosaic will multiply actively in growing isolated root tips for periods of at least 25 and 30 weeks, respectively. It may be presumed that they can be so maintained indefinitely. The cultures can be kept under strict physiological control, are entirely free from danger of accidental contamination with other viruses, and require comparatively little space. As a means of maintaining stock cultures of viruses, the method appears to present many advantages over methods previously in use. It would, of course, be dangerous to conclude that all plant-disease viruses can be so cultivated.

The absence of symptoms of disease in the roots is worthy of note. It seems possible that this may have its explanation in the chlorophyll-free character of the host tissue used. The pathological picture ordinarily associated with these diseases, the mottling, distortion, and stunting of the aerial parts of the plant, may be referred to the effects of the disease on the chlorophyll apparatus or to disturbances in metabolism resulting from injury to that apparatus. The roots used in these cultures were apparently free of chlorophyll. Photosynthesis, if occurring at all, was reduced to a very low level, far below that necessary for the maintenance of growth (30), but the requisite product of photosynthesis was supplied to the roots in adequate amounts in the form of sucrose added to the nutrient. The roots were thus freed from dependence on photosynthesis and hence from any injury that might be due to disturbance of this process. The fact that under the conditions described the roots showed no ill effects, although carrying considerable quantities of actively multiplying virus, would seem to suggest that the pathogenicity of these viruses is largely due to specific effects on the process of photosynthesis rather than to injurious effects on tissues in general. The increase of virus in these roots makes the hypothesis, sometimes advanced, that multiplication of virus is dependent on the chlorophyll apparatus, seem untenable.

The comparative ease of controlling the experimental conditions under which the viruses multiplied in these cultures adds considerably to the importance of the method. All of the factors constituting the effective environment, insofar as they are understood, can be controlled (30, 31). The method seems favorable for use in studies on the effects of temperature and light on the growth of viruses. The mineral nutrition may be varied in a much simpler manner than is possible when entire plants are used. The organic nutrition of the host tissues can be more easily controlled than by any other method at present available, since the normal products of photosynthesis can be varied at will without at the same time altering the external conditions that usually control their production.

The cultivation of the viruses of tobacco mosaic and aucuba mosaic in growing isolated root tips is of interest from another point of view. During the last decade, a number of viruses of animal diseases, notably those of foot-and-mouth disease (Hecke, 16, 17, 18; Maitland and Maitland, 21), herpes (Andrewes, 1, 3; Haagen, 14; Parker, 22; Parker and Nye, 24; Rivers, Haagen, and Muckenfuss, 27), myxoma (Haagen, 15; Benjamin and Rivers, 4), vaccinia (Carrel and Rivers, 9; Li and Rivers, 19; Maitland and Laing, 20; Rivers and Ward, 25; Rivers, Haagen, and Muckenfuss, 26, 27, 28; Parker and Nye, 23), variola (Haagen, 13, 14), "Virus III" (Andrewes, 1, 2), fowl-pox (Findlay, 12), vesicular stomatitis (Carrel *et al.*, 10), Rous sarcoma (Carrel, 5, 6, 7; Carrel and Ebeling, 8), pseudorabies (Traub, 29), the common cold (Dochez *et al.*, 11), and others, have been multiplied in cultures to which living tissues have been added. In most cases, however, the media employed have been unsuitable for the indefinite multiplication of the host tissues. The viruses have, therefore, been transferred at each passage to fresh pieces of surviving tissue. In fact, the usual practice has been to use as inoculum not a fragment of infected tissue, but a small amount of supernatant after it has been freed of cells by centrifuging or by filtration. The nutrient used in cultures of plant viruses, while adequate for indefinite multiplication of the host tissues, is also simpler than that commonly used in cultures of animal viruses, since it contains no serum, broth, embryonic juice, tissue extract, or other complex organic constituents, with the exception of a very small amount of yeast extract.

SUMMARY

A method has been developed for the cultivation of the viruses of tobacco mosaic and aucuba mosaic in isolated root tips grown *in vitro*. The viruses have multiplied actively under controlled, aseptic conditions, in the apparent absence of chlorophyll, and without the production of obvious symptoms. The method provides a simple means of maintaining stocks of these two viruses in a state of active multiplication, yet free from danger of contamination with other viruses.

LITERATURE CITED

1. ANDREWES, C. H. Virus III in tissue cultures. I. The appearance of intranuclear inclusions in vitro. Brit. Exp. Path. **10:** 188–190. 1929.
2. ——————. Virus III in tissue cultures. II. Further observations on the formation of inclusion bodies. III. Experiments bearing on immunity. Brit. Jour. Exp. Path. **10:** 273–280. 1929.
3. ——————. Tissue culture in the study of immunity to herpes. Jour. Path. and Bact. **33:** 301–312. 1930.
4. BENJAMIN, B., and T. M. RIVERS. Regeneration of *virus myxomatosum* (Sanarelli) in the presence of cells of exudates surviving in vitro. Proc. Soc. Exp. Biol. and Med. **28:** 791–792. 1931.
5. CARREL, A. Action de l'extrait filtré du sarcome de Rous sur les macrophages du sang. Compt. Rend. Soc. Biol. **91:** 1069–1071. 1924.
6. ——————. Effets de l'extrait de sarcome fusocellulaire sur des cultures pures de fibroblastes. Compt. Rend. Soc. Biol. **92:** 477–479. 1925.
7. ——————. Some conditions of the reproduction in vitro of the Rous virus. Jour. Exp. Med. **43:** 647–668. 1926.
8. ——————, and A. H. EBELING. The transformation of monocytes into fibroblasts through the action of the Rous virus. Jour. Exp. Med. **43:** 461–468. 1926.
9. ——————, and T. M. RIVERS. La fabrication du vaccin in vitro. Compt. Rend. Soc. Biol. **96:** 848–850. 1927.
10. ——————, P. K. OLITSKY, and P. H. LONG. Multiplication du virus de la stomatite vésiculaire du cheval dans les cultures de tissus. Compt. Rend. Soc. Biol. **98:** 827–828. 1928.
11. DOCHEZ, A. R., K. C. MILLS, and Y. KNEELAND. Cultivation of the virus of the common cold in tissue medium. Proc. Soc. Exp. Biol. and Med. **29:** 64–66. 1931.
12. FINDLAY, G. M. A note on the cultivation of the virus of fowl-pox. Brit. Jour. Exp. Path. **9:** 28–29. 1928.
13. HAAGEN, E. Ueber das Verhalten des Variola-Vakzinevirus in der Gewebekultur. Centralbl. Bakt., Abt. 1, Orig. **109:** 31–41. 1928

14. HAAGEN, E. Weitere Untersuchungen über das Verhalten des Variola-Vakzinevirus und des Herpesvirus in der Gewebekultur. Centralbl. Bakt., Abt. 1, Orig. **120**: 304–310. 1931.

15. ———. Untersuchungen über die übertragbare Myxomatose beim Kaninchen mit besonderer Berücksichtigung der Züchtung des Myxomavirus in der Gewebekultur. Centralbl. Bakt., Abt. 1, Orig. **121**: 1–9. 1931.

16. HECKE, F. Züchtungsversuche des Maul- und Klauenseuchevirus in Gewebekulturen. Centralbl. Bakt., Abt. 1, Orig. **116**: 386–414. 1930.

17. ———. Weitere Mitteilungen über die künstliche Vermehrung des Maul- und Klauenseuchevirus in Gewebekulturen. Centralbl. Bakt., Abt. 1, Orig. **119**: 385–397. 1931.

18. ———. Züchtung des Maul- und Klauenseucheerregers. Centralbl. Bakt., Abt. 1, Ref. **102**: 283–284. 1931.

19. LI, C. P., and T. M. RIVERS. Cultivation of vaccine virus. Jour. Exp. Med. **52**: 465–470. 1930.

20. MAITLAND, H. B., and A. W. LAING. Experiments on the cultivation of vaccinia virus. Brit. Jour. Exp. Path. **11**: 119–126. 1930.

21. MAITLAND, M. C., and H. B. MAITLAND. Cultivation of foot-and-mouth disease virus. Jour. Comp. Path. and Therapy **44**: 106–113. 1931.

22. PARKER, F. The cultivation of vaccine virus in vitro. Jour. Med. Res. **44**: 645–650. 1925.

23. ———, and R. NYE. Studies on filterable viruses. I. Cultivation of vaccine virus. Am. Jour. Path. **1**: 325–335. 1925.

24. ———, and ———. Studies on filterable viruses. II. Cultivation of herpes virus. Am. Jour. Path. **1**: 337–340. 1925.

25. RIVERS, T. M., and S. M. WARD. Observations on the cultivation of vaccine virus in lifeless media. Jour. Exp. Med. **57**: 51–63. 1932.

26. ———, E. HAAGEN, and R. S. MUCKENFUSS. A method of studying virus infection and virus immunity in tissue cultures. Proc. Soc. Exp. Biol. and Med. **26**: 494–496. 1929.

27. ———, ———, and ———. Development in tissue cultures of the intracellular changes characteristic of vaccinal and herpetic infections. Jour. Exp. Med. **50**: 665–672. 1929.

28. ———, ———, and ———. A study of vaccinal immunity in tissue cultures. Jour. Exp. Med. **50**: 673–685. 1929.

29. TRAUB, E. Cultivation of pseudorabies virus. Jour. Exp. Med. **58**: 663–681. 1933.

30. WHITE, P. R. Influence of some environmental conditions on the growth of excised root-tips of wheat seedlings in liquid media. Plant Physiology **7**: 613–628. 1932.

31. ———. Concentration of inorganic ions as related to growth of excised root-tips of wheat seedlings. Plant Physiology **8**: 489–508. 1933.

32. ———. Unlimited growth of excised tomato root-tips in a liquid medium. Plant Physiology **9**: ———. 1934. (In press.)

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